# GENOTOXIC EFFECTS OF NANO AND BULK FORMS OF ASPIRIN AND IBUPROFEN ON BLOOD SAMPLES FROM PROSTATE CANCER PATIENTS COMPARED TO THOSE FROM HEALTHY INDIVIDUALS.

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# Genotoxic effects of nano and bulk forms of aspirin and ibuprofen on blood samples from prostate cancer patients compared to those from healthy individuals.

The protective effects of NSAIDs against oxidative damage, quantification of DNA repair capacity and major signal transduction pathways in lymphocytes from healthy individuals and prostate cancer patients

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# Abstract

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Keywords, aspirin, ibuprofen, repair of DNA damage, oxidative stress, P53, ATM and ATR.

Inhibiting inflammatory processes or eliminating inflammation represents a logical role in the suppression and treatment strategy of cancer. Several studies have shown that anti-inflammatory drugs (NSAIDs) have promise as anticancer agents while reducing metastases and mortality. NSAIDs are seriously limited by side effects and their toxicity, which can become cumulative with their long-term administration for chemoprevention. The huge development in nanotechnology allows the drugs to exhibit novel and significantly improved properties compared to the large particles of the respective bulk compound, leading to more targeted therapy and reduced dosage. The overall aim of this thesis is to add to our understanding of cancer prevention and treatment through studying the genotoxicity mechanisms of NSAIDs agents in lymphocytes. In this study, the genotoxicity mechanisms of NSAID in bulk and nanoparticles forms a strategy to prevent and minimise the damage in human lymphocytes. Aspirin nano (ASP N) caused a significant decrease in deoxyribonucleic acid (DNA) damage compared to aspirin bulk (ASP B). Also, ibuprofen nano (IBU N) showed a significant reduction in DNA damage compared to ibuprofen bulk (IBU B). Micronuclei (MNi) decreased after ASP N, ASP B and IBU N in prostate cancer patients and healthy

individuals, and the ibuprofen bulk showed a significant increase of MNi formation in lymphocytes from healthy and prostate cancer patients when compared to untreated lymphocytes from prostate cancer patients. In order to study the geno-protective properties of these drugs, the protective effect of NSAIDs and the quantification of the DNA repair capacity in lymphocytes was studied. ASP N was found to increase the DNA repair capacity and reduced the reactive oxygen species (ROS) formation significantly more than ASP B. Finally, the role of NSAIDs on some key regulatory signal transduction pathways in isolated lymphocyte cells was investigated by studying their effect on ataxia-telangiectasia-mutated kinase (ATM) and ataxia-telangiectasia and Rad3-related kinase (ATR) mRNA. ATM mRNA significantly increased after treatment with ASP B, ASP N and IBU N. ATR expression also increased after treatment with IBU B and IBU N, but was only significant with IBU N. These findings indicate that a reduction in particle size had an impact on the reactivity of the drug, further emphasising the potential of nanoparticles as improvement to current treatment options.

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# List of Abbreviations

**AID:** Activation-Induced Deaminase

**ALS:** Alkali Labilesite

**α:** alpha

**APS:** Ammonium Persulphate

**ANOVA:** Analysis of Variance

**ASP B:** Aspirin Bulk

**ASP N:** Aspirin Nano

**ATM:** Ataxia-Telangiectasia-Mutated kinase (ATM)

**A-T:** Ataxia-Telangiectasia

**ATR:** Ataxia-Telangiectasia and Rad3-related kinase (ATR)

**BER:** Base Excision Repair

**BiNC:** Binucleated

**BLM:** Bleomycin

**BSA:** Bovine Serum Albumin

**$CO_2$:** Carbon Dioxide

**CCD:** Charge Coupled Device

**Chk2:** Checkpoint Kinase 2

**COX-1:** Cyclooxygenase enzyme 1

**COX-2:** Cyclooxygenase enzyme 2

**CDKs:** Cyclin-Dependent Kinases

**CDK:** Cycline Dependent Kinase

**Cyto-B:** Cytochalasin-B

**CBMN:** Cytokinesis-Block Micronucleus Assay

**CT:** Threshold Cycle**.**

**DC:** Dendritic Cells

**DNA:** Deoxyribonucleic Acid

**DTT:** Dithiothreitol

**DCF:** 2', 7'–Dichlorofluorescein

**DCFDA:** 2', 7'–Dichlorofluorescein diacetate

**DMSO:** Dimethyl Sulphoxide

**DSB:** Double Strand Break

**DLS:** Dynamic Light Scattering

**DRP:** DNA Repair Percentage

**EDTA:** Ethylene Diamine Tetraacetic Acid

**FBS:** Fetal Bovine Serum

**G1 and G2:** Gap phase 1 and Gap phase 2

**GI:** Gastrointestinal

**HGF:** Hepatocyte Growth Factor

**Ku:** Heterodimer

**HGPIN:** High-Grade PIN

**HRR:** Homologous Recombination Repair

**HRP:** Horseradish Peroxidase

**HCR:** Host Cell Reactivation Assay

**$H_2O_2$:** Hydrogen peroxide

**HPMC:** Hydroxypropyl Methylcellulose

**IBU B:** Ibuprofen Bulk

**IBU N:** Ibuprofen Nano

**Ig:** Immunoglobulin

**IL 1:** Interleukin-1

**KDa:** Kilo-Dalton

**KCl:** Potassium Chloride

**LMP:** Low Melting Point Agarose

**LUTS:** Lower Urinary Tract Symptoms

**MMP:** Matrix Metalloproteinase

**MNi:** Micronucleus

**MN:** Micronuclei

**µg:** Micro-gram

**µl:** Micro-litre

**µM:** Micro-molar

**Ml:** Millilitre

**MMC:** Mitomycin C

**M phase:** Mitosis

**MoNC:** Mononucleated Cells

**MultiNC:** Multinucleated Cells

**NADPH:** Nicotinamide Adenine Dinucleotide Phosphatase

**NaCl:** Sodium Chloride

**$Na_2EDTA \cdot 2H_2O$:** Ethylenediaminetetraacetic acid disodium salt dihydrate

**NaOH:** Sodium Hydroxide

**NBUDs:** Nuclear Buds

**NC:** Negative Control

**NDI:** Nuclear Division Index

**NE:** Neuroendocrine Cells

**NER:** Nucleotide Excision Repair

**NF-κB:** Nuclear transcription factor kappa B

**NHEJ:** Non-Homologous End-Joining

**NMP:** Normal Melting Point Agarose

**NPs:** Nanoparticles

**NPBs:** Nucleoplasmic Bridge

**NSF:** Neuron-Specific Enolase

**NSAIDs:** Non-Steroidal Anti-Inflammatory Drugs

**$^1O_2$:** Singlet oxygen

**O2•−:** Superoxide anion

**OD:** Optical Density

**$^{\bullet}$OH:** Hydroxyl radical

**8-OHdG:** 8-Hydroxy deoxyguanosine

**PAGE:** Polyacrylamide gel

**PAP:** Prostatic Acid Phosphatase

**PBS:** Phosphate Buffer Saline

**PBLs:** Peripheral Blood Lymphocytes

**P53:** Tumour suppressor protein

**PCa:** Prostate Cancer

**PC:** Positive Control

**2-deoxyribose:** Sugar group

**PE:** Prostaglandin

**PGHS:** Prostaglandin endoperoxide H Synthase

**PGE2:** Prostaglandin E2

**PHA:** Phytohaemagglutinin

**PIA:** Proliferative Inflammatory Atrophy

**PIN:** Prostatic Intraepithelial Neoplasia

**PIA:** Proliferative Inflammatory Atrophy

**PSA:** Prostate-Specific Antigen

**RAG:** Recombination-Activating Gene

**RNA:** Ribonucleic Acid

**ROS:** Reactive Oxygen Species

**RPA:** Replication Protein-A

**RPMI:** Roswell Park Memorial Institute

**Rpm:** Revolutions per minute

**RSS:** Recombination Signal Sequence

**RT:** Room Temperature

**SD:** Standard Deviation

**SDS:** Sodium Dodecyl Sulphate

**SE:** Standard Error of the mean

**SCGE:** Single Cell Gel Electrophoresis

**SHM:** Somatic Hypermutation Diversity

**SOD:** Superoxide Dismutase

**S phase:** Synthesis phase

**SPSS:** Statistical Package for Social Sciences

**SSBs:** Single-Strand Breaks

**TA:** Transient Amplifying cells

**TBS-T:** Tris Buffer Solution with Tween-20

**T-BHP:** Tertiary-butyl hydroperoxide

**TCR:** T cell Receptors

**TEMED:** N, N, N', N' Tetramethylethylenediamine

**TEM:** Transmission Electron Microscopy

**TNF-α:** Tumour Necrosis Factor alpha

**UDS:** Un-programmed DNA Synthesis

**UV:** Ultraviolet Radiation

**V (D) J:** Variable Diverse-Joining

**VEGF:** Vascular Endothelial Growth Factor

**V/v:** Volume per volume

**XLF:** XRCC4-like Factor

**XP:** Xeroderma Pigmentosum

**XRCC3:** X-ray-Repair Cross-Complementing 3

# Chapter 1

# General Introduction

# 1 Introduction

Prostate cancer (PCa) is the most frequent malignancy among men in economically developed countries. The development of prostate cancer involves a set of cellular pathways in its initiation and progression, which can be used as a target for chemoprevention and the treatment of cancer. For instance, using androgens as chemo-preventive agents has been confirmed to decrease the risk of prostate cancer (Jafari et al., 2009; Wang et al., 2013). There is growing evidence that an inflammatory pathway is involved in the regulation of cellular events in prostate cancer (Thapa and Ghosh, 2015). This has led to the hypothesis that aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) may be associated with a reduced risk of cancer.

## 1.1 Prostate Anatomy

The prostate is a walnut-shaped exocrine gland. It is located just under the bladder and surrounds the urethra. Just behind the prostate are the seminal vesicles, which make the fluid for semen. The vas deferens carries sperm from the testes to the seminal vesicles. The urethra runs through the middle of the prostate, from the bladder to the tip of the penis, and has two functions: urination and ejaculation. The portion of the urethra that runs through the prostate is called the prostatic urethra. During ejaculation, the muscular component of the prostate squeezes the fluid into the urethra, and it is expelled with sperm as semen (Figure 1) (Marieb and Koehn, 2015; Scott and Fong, 2013).

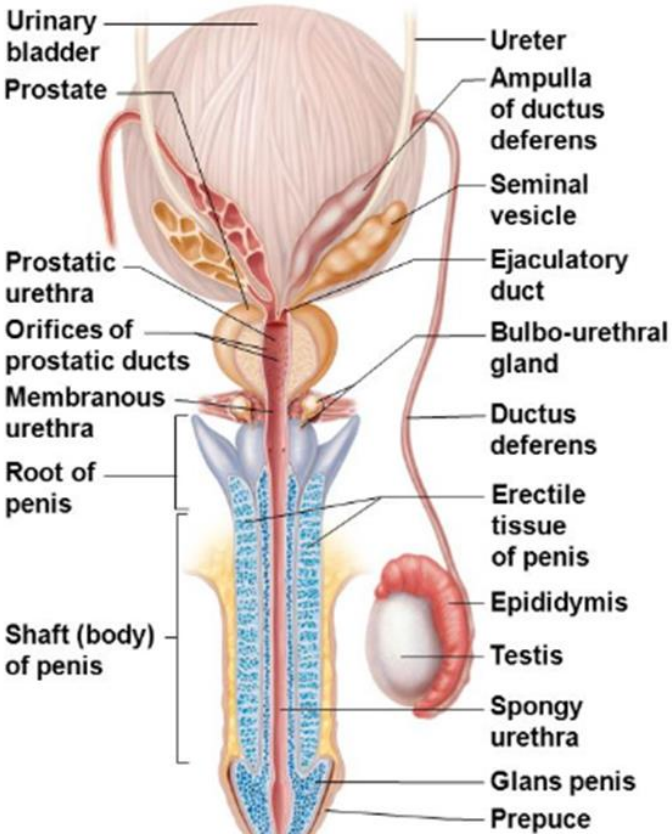



Figure 1. Prostate anatomy (Marieb and Koehn, 2015).

### 1.1.1 Prostate Structure

The human prostate can be classified in two different ways: zones or lobes. First, the zone classification is usually used in pathology. In this classification system, the human prostate gland is divided into several morphological zones: the peripheral zone, the central zone and the transition zone. Each zone can harbour different prostatic diseases. For instance, most prostate carcinomas develop in the peripheral zone (Bhavsar and Verma, 2014; Cunha et al., 1987). In contrast, benign prostatic hyperplasia (BPH), which is common in older men, arises from the transition zone.

Second, another way to classify the prostate is by lobes; this classification system also divides the prostate into four distinct regions: the anterior lobe, the posterior

lobe, the lateral lobes and the median or middle lobe. This classification is usually common in the anatomy of the prostate (Aaron et al., 2016).

### 1.1.2 Prostate Function

The main purpose of the prostate gland is to produce an alkaline secretion that forms an important component of semen. The alkaline nature of semen can extend the lifespan of spermatozoa. The major components of prostatic secretion are prostate-specific antigen (PSA), citrate (18.7 mg/ml), zinc (488 µg/ml), spermine (243 mg/ml) and cholesterol (78 mg/ml) (Franz et al., 2013).

## 1.2 Histology of Prostate Cancer

A normal prostate is composed of two types of cells known as epithelial and stromal cells (Krušlin et al., 2015). The epithelial cell layer consists of four main cell types: basal cells, transient amplifying (TA) cells, luminal epithelial cells and neuroendocrine (NE) cells, which have been identified through their location and morphology and by using different marker expressions (Prajapati et al., 2013). The basal cell layer is in close contact with the basement membrane containing the stem cells that are responsible for the proliferative compartment of the prostate epithelium. The basal cells express p63 (a homolog of the tumour suppressor gene p53), cytokeratin (CK) 5 and 14, cluster differentiation (CD) 44, Bcl-2 (an anti-apoptotic factor), a hepatocyte growth factor (HGF) and have reduced or absent expression of androgen receptor (AR). The lack of AR indicates that the basal cells are not dependent on androgens for survival (Heer, 2011)

The epithelium is divided into two different layers. The basal layer is made up of cuboidal epithelial cells, and luminal cells are the prostate's primary cell type,

which shapes a layer of columnar secretory cells whose main role is the production of prostate-specific antigen (PSA), prostatic acid phosphatase (PAP) and kallikrein-2, which are involved in seminal fluid secretion. Also, the luminal cells are androgen independent and can express a low molecular weight CK8 and 18, CD57 and p27Kip1 (a cell cycle inhibitor) (Heer, 2011). Occasionally, NE cells can be found spread in the basal and luminal layers of the prostate. NE cells are androgen-insensitive cells and can express chromogranin A, synaptophysin and neuron-specific enolase (NSF). Also, they can produce and secrete neuropeptides, such as bombesin, calcitonin, and neurotensin, which are believed to support epithelial cell growth and differentiation (Prajapati et al., 2013) Furthermore, the TA cells differentiate into terminal end-stage secretory luminal cells. These cells then give rise to heterogeneous subpopulations of cells through cell migration from the basal layer into the luminal layer and have a central role in the development of all epithelial cell forms in the prostate. TA cells can express both basal as well as luminal cell markers (CK5, CK8, CK14, CK18, AR and PSA) (Karthaus et al., 2014).

## 1.3 Prostate Carcinogenesis

Cancer can be defined as abnormal growth of cells (Gabriel, 2007; Hanahan and Weinberg, 2011). Despite all cell types having the potential to develop malignant alterations leading to cancer, only the epithelial cells can become carcinomas. The cell cycle is interrupted, and the new tumour cells hyperproliferate in a local area before being able to spread and invade surrounding tissue regions and eventually all parts of the body through both the lymphatic and vascular system (Lowengrub et al., 2010).

In carcinogenesis, healthy cells are converted into cancer cells as a result of unlimited cell division. The central role of normal cells is to form an epithelium and to maintain a balance between proliferation and cell death with tightly regulated processes. Deoxyribonucleic acid (DNA) mutations can cause disturbances to these processes, leading to much more rapid cell division than normal cells, and therefore, can have much higher rates of proliferation (Ayala and Ro, 2007). Prostate cancer is believed to develop from early proliferative inflammatory atrophy (PIA) a predicted risk factor lesion to prostate cancer and prostate intraepithelial neoplasia (PIN) via the accumulation of genetic aberrations in epithelial cells (Shen and Abate-Shen, 2010). A basal cell origin for PCa has been suggested as human prostate basal cells expressing marker proteins, including AR and PSA (Rybak et al., 2015).

### 1.3.1 Prostatic Intraepithelial Neoplasia (PIN)

PIN is a primary precursor of human prostate cancer. PIN is generally characterised by the appearance of luminal epithelial hyperplasia, reduction in basal cells, enlargement of nuclei and nucleoli, cytoplasmic hyperchromasia and nuclear atypia (Shah and Zhou, 2012). PIN can be classified into low grades (grade 1) and high grades (grades 2 and 3). Low-grade PIN is characterised by crowded and irregularly spaced epithelium cells with elongated hyperchromatic and variably sized nuclei (Ayala and Ro, 2007).

It is difficult to differentiate low-grade PIN from the normal and hyperplastic epithelium. High-grade PIN (HGPIN) is widely considered to be the precursor of prostate cancer. HGPIN is characterised histologically by an overgrowth of epithelial cells with cytological alterations representing carcinomas, such as

enlarged nuclei with prominent nucleoli, more noticeable epithelial cell crowding and stratification (Jung et al., 2016).

### 1.3.2 Benign Prostatic Hyperplasia

Benign prostatic hyperplasia (BPH) is referred to as benign prostatic hypertrophy. The word hyperplasia is derived from the ancient Greek word hyper and plasia, meaning excess growth in the amount of organic tissue. BPH is a condition characterised by slow progressive enlargement of the prostate gland and histologically associated with the deregulated proliferation of both epithelial and stromal cells. This noncancerous overgrowth of the prostate cells can exert pressure on the urethra and cause an obstacle to urine flow and lower urinary tract symptoms (LUTS) (Izumi et al., 2013).

Moreover, BPH is considered the main cause of LUTS in elderly males. Despite its obvious impact on health, very little is known about the biological processes and causes of the development of BPH. A number of theories have been suggested to explain the aetiology of the pathogenesis of BPH, including abnormal proliferation and apoptosis of stem cells, hormonal imbalance, apoptosis, epithelial mesenchymal transition, embryonic awakening and inflammation (Notara and Ahmed, 2012)

## 1.4 Molecular Changes of Prostate Cancers

Since most human genomes have large quantities of DNA sequences that do not code for proteins, protein-coding DNA sequences make up approximately 1.5% of the genome, and most mammalian genes are divided into introns (noncoding) and exons (coding) (Alberts et al., 2017; Kellis et al., 2014). Only mutations in the exon region will have an effect on the amino acid sequences that compose

proteins. Although the genetic causes of prostate cancer remain poorly understood, we know that cancers are derived from single somatic cells (Stergachis et al., 2013). The cells in the emerging neoplasm undergo a series of genetic changes that lead to alterations in gene activity and phenotype and, therefore, lead to cancer development (Chatterjee et al., 2017; Ponder, 2001). Cancer results from mutations that affect genes involved in the regulation of cellular growth or death (Hanahan and Weinberg, 2011).

There are many different types (and subtypes) of cancer, and their complexity makes it difficult to trace a particular tumour's origin. The past three decades have seen a significant rise in biochemistry and cellular and molecular biology that aid the understanding of cell cycle processes that involve the transformation of a normal cell to a cancerous cell.

Most of the cancer cell genotypes arise from a manifestation of six essential alterations in the cell physiology that collectively dictate tumour development: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, infinite replication and the ability to invade tissue and metastasise (Hanahan and Weinberg, 2011).

Normal cells need external growth signals (growth factors) to stimulate cell growth and division. These signals are received by a receptor molecule on the cell surface. Cancer cells, however, can divide without external stimuli by generating their own growth signals. The second feature is the sensitivity of antigrowth signals; the growth of normal cells is controlled by growth inhibitors on the surfaces of neighbouring cells. These inhibitors interrupt cell division in the interphase; in contrast, cancer cells do not receive signals to inhibit their growth. The third capability is the acquired capability of sustained growth.

Normally, cells divide on average about 50 to 70 times, which is influenced by the length of telomeres. Telomeres are repetitive regions at the end of chromosomes that act as buffers to prevent the loss of DNA as a result of incomplete semi-conservative replication. Each time a cell divides, part of the telomere is lost during DNA replication. If the telomeres get too short, the cell will either enter cellular senescence or undergo apoptosis. Cancer cells can replicate without control and are able to lengthen their telomeres constantly.

The next capability is evasion of apoptosis. Apoptosis is a major mechanism of cancer control, and it is exerted by the p53 tumour suppressor protein in response to DNA damage. In order for cancer to progress, the p53 gene is mutated, and consequently, apoptosis does not proceed normally. Angiogenesis is the creation of new blood vessels that are formed in response to growth factors secreted by stromal cells and cancer cells. These processes are required to supply oxygen and nutrients to the proliferating tumour cells. Angiogenesis plays a vital role in the growth and spread of cancer (Ziyad and Iruela-Arispe, 2011).

Morever, the next feature is tissue invasion and metastasis, where cancer cells spread to the surrounding tissue and distant organs. Cancer metastasis is the spread of cancer cells to tissues and organs beyond where the tumour originated and the formation of a new tumour (Valastyan and Weinberg, 2011).

## 1.5 Tumour Suppressor Genes and Oncogenes

Cancer genes are grouped into three main classes: oncogenes, tumour suppressor genes and DNA repair genes. Oncogenes are considered to be cancer causing genes, as they activate cellular proliferation and can lead to differentiation and unregulated cell growth (Agarwal et al., 2013; Ponder, 2001).

Most oncogenes arise from altered versions of the genes known as a proto-oncogene responsible for normal cell growth and division. Most oncogenes are usually dominant mutations that involve a rise in protein activity, an increase in protein concentration or chromosomal translocation, causing expression of a different cell type (Gabriel, 2007; Lee and Muller, 2010).

Tumour suppressor genes are also known as anti-oncogenes. Their function in normal cells is to regulate cellular proliferation. Mutation in these genes results in a loss of gene function, which promotes carcinogenesis. They are generally dominant, and common mutations include an increase in protein activity or loss of regulation, increase in protein concentration or chromosomal translocation, causing gene expression of a different cell type (Leemans et al., 2011).

## 1.6 DNA and Chromatin

A replica of our genetic information is kept well-preserved in the nuclei of the cell and is organised into chromosomes, which carry heritable material in the long molecules of DNA. DNA is made of repeated units called nucleotides consisting of three functional groups: a sugar group (2-deoxyribose), a phosphate group and a nitrogen base attached to each sugar. The nucleotides are linked together in a chain through a sugar group and a phosphate group, creating long DNA strands with bases. Two DNA chains are held together by hydrogen bonds between bases, and portions of the nucleotides hold the two strands together, creating a double helix.

Each DNA strand serves as a pattern or template to specify the sequence of nucleotides in which a new complementary strand passes on the hereditary material to the next generation (Alberts, 2017).

Our cellular DNA is subjected to constant assaults from both externally and internally generated damaging agents. It is estimated that each of the ~$10^{13}$ cells in the human body is assaulted by tens of thousands of DNA lesions per day (Cohen et al., 2015). Most of these occasional lesions are raised via physiological processes, such as reactive oxygen species compounds generated during a normal aerobic metabolism process or through redox-cycling actions involving environmental toxins and Fenton reactions mediated by heavy metals (Ienco et al., 2011).

DNA damage can also generated by errors in DNA replication during DNA synthesis, and also by exogenous factors, such as genotoxic chemicals and ionising radiation (Ciccia and Elledge, 2010). Unrepaired DNA damage can cause rearrangements and translocation of the chromosome, which can lead to cell death and cancer. For Survival, mammalian cells are equipped with sophisticated DNA repair mechanisms to detect and repair different forms of DNA damage (Broustas and Lieberman, 2014).

### 1.6.1 DNA Damage

Based on the type of damage sources, a variety of DNA damage is generated. The simple form of endogenous DNA damage is a result of the aqueous environment around the DNA, by the hydrolysis of the N-glycosidic bond between the DNA bases and, therefore, isolating the nucleobase from the deoxyribose, thus leaving an apurinic/apyrimidinic (AP) site. Another common reaction involving hydrolysis is the deamination of DNA bases carrying exocyclic amino groups. The most frequent of these lesions is the conversion cytosine to uracil, which will finally lead to mutations (Alberts, 2017).

DNA is also subject to chemical alterations by molecular oxygen that is produced during normal cellular metabolism. Among the most vital of these molecules are reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radicals. ROS are produced endogenously from leaking electrons from the respiratory chain in the mitochondria; therefore, during the transition, a metal-mediated decrease of oxygen or hydrogen peroxide, and during synthesis of prostaglandin and leukotrienes, ROS can be highly oxidative to the guanine resulting in the generation of 8-oxo-guanine (Birben et al., 2012).

ROS can cause > 100 types of oxidative DNA adducts, such as single-strand breaks (SSBs), double-strand breaks (DSBs), chemical modifications of the bases and sugar, and DNA protein cross-linking. Exogenous agents, such as ultraviolet radiation (UV) and ionising radiation, can penetrate and damage the human genome by inducing different DNA lesions, including the production of cyclobutane pyrimidine dimers that, if not repaired or are misrepaired during the replication, can also lead to mutations. Adenine and guanine can be spontaneously deaminated to produce hypoxanthine and xanthine, respectively (Broustas and Lieberman, 2014).

### 1.6.2 DNA Double-Strand Breaks (DSBs)

A DNA double-strand (DSB) results from the breaking of the phosphodiester bonds between the sugar backbones on both DNA strands, which occurs with a region of 10–20 base pairs (bp) in the same or opposite DNA strands (Sutherland et al., 2002). Only 1–10 DSBs is sufficient to induce p53-dependent arrest and cell death (Nowsheen and Yang, 2012). A DSB is considered to be the most cytotoxic form of DNA lesion and is used in cancer therapy to kill cancer cells (Jekimovs et al., 2014). SSBs, in contrast, result from the break of the

phosphodiester bond between two neighbouring deoxyribose residues in the backbone of DNA. SSBs are eliminated with a half-life of minutes; using a copy of the genome as a template to reconstruct the damaged copy, intact DNA being utilised to guide the repair. Single-strand DNA damage is consequently not very toxic, and in some cases, more than 100,000 SSBs are required to promote cell cycle arrest (Polo and Jackson, 2011).

DSBs cannot be repaired through the template-directed repair system pathway. Therefore, the DSBs can be repaired by two major mechanisms: the DSB ends can be directly ligated together with little or no sequence homology between both broken ends, known as nonhomologous end joining (NHEJ), or repaired by homologous recombination (HR) using an undamaged DNA template on the sister chromatid to repair the break (Ciccia and Elledge, 2010).

DSBs can be generated exogenously by ionising radiation or many chemotherapeutic drugs, such as bleomycin and etoposide, and endogenously as physiological processes like oxidative respiration that generate ROS, Variable Diverse-Joining (V(D)J) recombination, class switch recombination (CSR), replication across a nick, meiotic recombination and at eroded telomeres (Hoeijmakers, 2009; Koster et al., 2007).

### 1.6.3 Endogenous DSBs

#### 1.6.3.1 V (D) J Recombination

V (D) J recombination is a site-specific recombination event that occurs specifically in the development of the lymphocytes and is initiated via induction of DNA double strands that are repaired by NHEJ. V (D) J is important for the diversity of the B- and T-cells and developing the immune system. Our immune response is helped enormously by two major cell types: B and T lymphocytes. B

lymphocytes secrete soluble antibodies each with specific antigen-binding site, to fight against foreign substances, while T lymphocytes express surface receptors—T cell receptors (TCR)—that recognise and react with antigens on the surfaces of the other cells. Antibodies are composed of two different kinds of polypeptide chains: two identical heavy chains and two identical light chains. Each polypeptide chain consists of a C terminal constant region end and an N terminal variable region end (Khan and Ali, 2017).

The range of the variable regions is created by V(D)J recombination that recombines numerous other altered gene segments into one continuous exon that encodes the variable N-terminal region of both the light and heavy chain. The T-cell receptor also has two designed alpha (α) and beta (β) chains, each containing variable (V) amino-terminal regions and a constant (C) region that similarly are assembled by the V(D)J recombination.

The variable region's diversity is generated at several different levels. First, each heavy chain has multiple copies of the gene regions V (variable), D (diverse) and J (joining) gene segments and can be recombined in multiple ways. Similarly, in the light chain, the variable regions have two recombination segments: V and J. Second, as the V(D)J recombination individually produces variable regions in both heavy and light chains in each B-cell, the arrangement of diverse heavy and light chains into one antibody leads to high diversity. Furthermore, NHEJ, a major repair pathway in mammalian cells that joins together cleaved coding regions during repair of V(D)J breaks, is error-prone and can lead to deletions and insertions of nucleotides, thus extra amplifying the diversity of the antibodies' antigen binding region (Roth, 2014).

There are two recombination-activating genes (RAG) products called RAG 1 and RAG 2 that initiate V(D)J recombination and work as a multi-subunit complex to induce a DSB between an antigen receptor coding segment and recombination signal sequence (RSS). Broken coding ends are covalently sealed in a hairpin structure, while single ends are blunt 5-phosphorylated molecules. Next, the broken molecules are ligated to shape single and coding joints. Single coding joints are joined precisely in a head-to-head fashion. Coding ends are ligated to form imprecise coding joints, thereby, introducing junctional diversity. The first phase of recombination is mediated by lymphoid-specific genes RAG 1 and RAG 2. The later phase of the reaction needs some factors, including many involved in DSB repair (Helmink and Sleckman, 2012).

#### 1.6.3.2 Class-Switch Recombination (CSR)

Upon exposure to the antigen, B cell~~s~~ immunoglobulin (Ig) genes undergo ~~some~~ DNA alteration events known as classic-switch recombination (CSR) and somatic hypermutation (SHM) to enhance antibody diversity. CSR rearranges the constant region by transferring the variable region to a constant region and can occur anywhere within the switch regions. Activation-induced deaminase (AID) is a key player in both processes by converting cytosine to uracil in single-strand DNA in a co-transcriptional manner. These mutations are removed by base excision and a mismatch repair pathway that leads to the formation of DSBs that enable class-switch recombination. In variable regions, the hypermutation occurs from a high frequency of errors during the repair of C–U deamination (Wiedemann et al., 2016).

#### 1.6.3.3 Meiotic Recombination

During meiosis, the recombination of the homologous chromosomes is carried out through a special pathway for the generation and repair of DNA DSBs induced by the endonuclease spo11 (Lu and Yu, 2015). Spo11 appears to act via a type II topoisomerase-like reaction to generate protein-linked DSBs (Khan and Ali, 2017). Spo11 dimers insert in a coordinated manner break both DNA strands, producing a DSB in which spo11 covalently binds to the newly created 5-DNA ends and the catalytic tyrosine residue in spo11. The spo11 protein must be removed by endonucleolytic cleavage, uncovering DSBs and a resection of 3'-ssDNA for the DSB to be repaired. Meiosis-specific recombinase Dmc1 binds with Rad51 and forms the nucleoprotein filaments that are needed for the homology search in the homologous chromosome (Lu and Yu, 2015).

## 1.7 DNA Damage Repair

Many defence mechanisms defend against spontaneous DNA damage. For example, the fitting of the DNA into the cell nucleus isolates it from mitochondrial oxygen consumption, and peroxisomes minimise contact with oxygen. Furthermore, packaging the DNA into chromatin protects against ROS contact. Ferritin and transferrin that are known as iron-containing substances that decrease ROS production (Pino et al., 2017). Also, several specialised enzymes, such as superoxide dismutase (SOD), peroxidases and peroxiredoxin proteins, work by limiting the exposure of DNA to ROS (Bhattacharyya et al., 2014). Cells also have an additional DNA repair mechanism that helps protect against DNA on a daily basis (Hurov et al., 2010).

Most of the DNA damaged agents, such as alkylating and ROS drugs, generate single-stranded DNA damage that leaves the opposite strand intact. This kind of

DNA damage is rapidly repaired by template-directed DNA repair systems, such as base excision repair (BER) and nucleotide excision repair (NER). In base excision repair, the single damaged bases are recognised and removed from the DNA molecules. The resulting short gap is then filled with the right base by newly synthesised DNA. NER, in particular, repairs bulky adducts, such as pyrimidine dimers and alkyl groups that severely distort the DNA helix.

The general process begins with excision of a short region of DNA lesions, and next, the remaining undamaged complementary strand acts as a template for synthesising the removed strand (Whitaker et al., 2017).

### 1.7.1 DNA Repair Mechanisms

To recover from the several types of DNA damage that occur, the cells have developed many mechanisms for repairing a different subgroup of lesions. At a minimum, most would agree that eukaryotic cells apply five major DNA repair mechanisms: BER, NER, mismatch repair (MMR) and DSB repair, which is repaired by both HR and NHEJ (Dexheimer, 2013).

### 1.7.2 Double-Strand Break Repair

The repair of the DSB is critical for maintenance of genome integrity and cell survival DNA (Khanna and Jackson, 2001; Srivastava and Raghavan, 2015; van Gent et al., 2001). In mammalian cells, the DSBs are repaired by two primary mechanisms: HR and NHEJ. These two repair pathways differ in their fidelity and the homologous template of DNA requirements of DSB repair. HR is considered to be the error-free repair mechanism because it employs the genetic information that is included in the identical sister chromatid that is available as a template for the recombination process. Contrary to NHEJ, HR is highly error-prone and

typically involves elimination of DSBs by direct ligation of DNA broken ends (Heyer et al., 2010; Lieber, 2010; San Filippo et al., 2008). NHEJ is predominant as a DSB repair pathway in mammalian cells that operate in all phases of the cell cycle. However, HR is restricted to the late mitosis S and G2 phases (Kass and Jasin, 2010). The basic mechanisms and the factors involved in these pathways are briefly outlined below.

### 1.7.3 Homologous Recombination (HR)

HR can be conceptually revealed in three separate phases: presynapsis, synapsis and postsynapsis. In the presynapsis phase, the DNA ends surrounding the DSB are initiated by a 5' to 3' end resection to produce molecules with 3' single-stranded tails (Zhang et al., 2013). The heterotrimeric MRN complex (Mre11–Rad50–Nbs1), together with CtIP (RBBP8), controls the initiation of resectioning in which the 5` ends on either side of the DSB are trimmed back to create 3` short overhangs of single-strand DNA (Liu and Huang, 2016).

The next step in the 5' to 3' resection is most likely continued by a joint action of the Bloom syndrome helicase (BLM, RecQ helicase-like) and Exol exonuclease (Nimonkar et al., 2008). Then, single-strand DNA is restricted by Replication protein-A (RPA) to eliminate the disruptive secondary structure, which is then the block binding of the RAD51 recombinase. RPA can be replaced later by RAD51 in combination with numerous recombination mediator proteins, for instance RAD52, BRCA2 and the RAD51 paralogues, including RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3 (Forget and Kowalczykowski, 2010). The resulting RAD51–ssDNA filament then performs the DNA sequence homology search, which is considered the central reaction of HR. When the homologous DNA has been recognised, RAD51 catalyses the DNA strand invasion reaction, followed

by invading the damaged DNA strand to the template DNA duplex (i.e., sister chromatid).

Next, DNA synthesis of the invading strand is then completed by DNA polymerase, which successively ligates them via DNA ligase I, yielding a four-way junction intermediate structure called a Holiday junction (Rastogi et al., 2010). The junction is a resolvase (MUS81-EME1/SLX1-SLX4/GEN1) into which both noncrossover or crossover yields by dissolving through a mechanism involving BLM-mediated branch migration and TOPOIIIα and RMI1-RMI2 (RecQ-mediated genome instability 1/2) by symmetrical cleavage of GEN1/Yen1 or Slx1/Slx4 or by asymmetric cleavage of the structure-specific endonuclease Mus81/Eme1, resulting in the error-free correction of the DSB (Croteau et al., 2014).

### 1.7.4 Non-Homologous End Joining (NHEJ)

NHEJ is a major DNA DSB repair pathway in a human cell system. All proteins that participate in NHEJ have a remarkable level of mechanistic flexibility compared with their counterparts in other repair processes, which allows NHEJ to produce different junction outcomes, although initiating from the same ends (Srivastava and Raghavan, 2015).

The initial stage in the NHEJ process requires recognition and binding of the heterodimer (Ku) that consist of Ku 70/ Ku 80 at both ends of the broken DNA molecules. Ku70/ku80 heterodimer adopts the shape of the ring that completely encircles duplex DNA (Davis and Chen, 2013). After binding Ku to DNA, the Ku-DNA complex attracts the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to produce the DNA-PK holoenzyme, which shows the protein kinase activity. Once bound to DNA, Ku can recruit DNA-PKcs and then move inward on

the DNA of Ku along the DNA, allowing DNA-PKcs to contact DNA termini (Bennett et al., 2012; Yoo and Dynan, 1999). Essentially, when the DNA-PKcs molecules bind with high affinity to opposing DSB ends, they form synapsis or tethering of the two DNA molecules. The DNA termini become available after autophosphorylation of DNA-PK (CS), which results from synapsis of DNA-PKcs (Thompson, 2012). Similar to most DNA repair processes, based on the complexity and type of the DSBs, DNA ends may need some alteration in advance of ligation. For instance, nonligatable DNA termini containing single-stranded overhang must be processed to become ligatable through DNA polymerase-mediated fill-in or nucleolytic reaction. During the NHEJ, the resynthesis of the missing nucleotides has been linked to the two members of the X family DNA polymerases: Pol μ and Pol λ (Ramsden, 2011). Instead, the effect of NHEJ-specific nuclease Artemis activities that include a DNA-PK independent 5-to-3 exonuclease activity, along with a DNA-PK-dependent endonuclease activity, which is obtained through phosphorylation by DNA-PK, can eliminate single-strand overhangs (Jeggo and O'Neill, 2002). Other enzymatic components that may be involved in the DNA end cleaning process include a number of the lesion-specific BER enzymes, such as Tdp1, APE1 and PNKP (Hegde et al., 2012), in addition to the two functional exonucleases Exo1 and WRN, which are altered in Werner syndrome patients (Bahmed et al., 2011; Perry et al., 2006). Therefore, the identical enzymes that play a part in the end-processing step of the NHEJ pathway are counted as being in control for overhang mispairing and involve either removal or addition of nucleotides that are associated with NHEJ-mediated repair. Next, either appropriately or sometimes inappropriately, DNA ligase IV achieves processing of ligation of the DNA end DNA termini in

conjunction with its binding partner XRCC4. Another factor, XRCC4-like factor (XLF), acts together with XRCC4-DNA ligase IV complex to promote DNA ligation (Gerodimos et al., 2017).

## 1.8 Non-Steroidal Anti-Inflammatory Drugs

NSAIDs are among the most commonly used over-the-counter medications worldwide with analgesic, antipyretic and anti-inflammatory effects. The use of NSAIDs have side effects, such as gastrointestinal (GI) complications, cardiovascular events and renal toxicity, which severely hinder their potential.

### 1.8.1 Aspirin

Aspirin, also known as acetylsalicylic acid, is one of the most widely used anti-inflammatory drugs. The active ingredient of aspirin, salicylic acid, was first recognised by the ancient Greek physician Hippocrates and came from the bark of certain willow trees (*Salix* spp.). However, aspirin was first chemically isolated and synthesised in its stable form by German chemist Felix Hoffmann in 1897 at the Bayer Pharmaceutical Company (Gensini and Conti, 2009). Aspirin possesses analgesic, anti-inflammatory, antiplatelet and antipyretic properties with an elimination half-life of about 15–20 minutes in plasma (Bruno et al., 2012). Besides the anti-inflammatory properties, aspirin can also prevent stroke and other cardiovascular diseases (Sutcliffe et al., 2013).

### 1.8.2 Ibuprofen

Ibuprofen was introduced in 1969 and its chemical name is 2-(4-isobutylphenyl)-propionic acid (Halford et al., 2012). It is another well-known NSAID. Similarly to aspirin, ibuprofen is a pain reliever that is used for the management of all types

of pain, inflammation and fever by suppressing the activity of the enzyme cyclooxygenase. Evidence has suggested other anti-inflammatory properties of ibuprofen, which include reduced cytokine production, inhibition of free radicals and signalling transduction, and modulation of leukocyte activity (Mazaleuskaya et al., 2015). Ibuprofen has also been found to help in treating Alzheimer's disease (Imbimbo et al., 2010; Matsuura et al., 2015). It has the least side effects, including GI complications, stomach ulcers and myocardial infarction, compared to the other NSAIDs (Harirforoosh et al., 2013).

### 1.8.3 Role of NSAIDs in Prostaglandin Synthesis

There is great evidence that indicates that NSAIDs exert their effect by inhibiting the activities of the cyclooxygenase enzymes, which is considered the key mechanism of NSAIDs' analgesic, antipyretic and anti-inflammatory properties (Meek et al., 2010; Vane, 1971). Inhibition of cyclooxygenase (COX) resulting in inhibition of prostaglandin and other eicosanoid synthesis mitigates pain, fever and inflammation.

COX is a key enzyme in the biosynthesis of prostaglandins that are derived from arachidonic acid (Day and Graham, 2013). The COX enzyme, also named prostaglandin endoperoxide H synthase (PGHS), is found in two isoforms of PGHS—PGHS-1 or COX-1 and PGHS-2 or COX-2—which are produced by different genes and both convert arachidonic acid to prostaglandin (see Figure 2) (Liu, 2011).

The COX-1 enzyme is constitutively expressed at high levels in particular cells and tissues, including platelets, monocytes, the endothelium, renal collecting tubules and seminal vesicles (Roth, 2011). In contrast, the COX-2 enzyme is produced at the site of inflammation and is known to be the predominant form in

the inflamed tissue, where it is induced by some cytokines., including interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF-α). There are a limited number of cells that can express COX-2 in response to mitogens, including endothelial cells, smooth cells, fibroblasts, monocytes, macrophages and synovial cells (Sokolove and Lepus, 2013).

NSAIDs can be classified into two categories, nonselective NSAIDs, which block both isoforms of COX enzymes, and selective NSAIDs (also called COX-2 inhibitors), which act by blocking the COX-2 enzymes rather than COX-1 and are less likely to cause an unfavourable gastrointestinal effect (Petersen and Nelson, 2010). Inhibition of COX-2 initiates the control of pain and inflammation (Ricciotti and FitzGerald, 2011). NSAID drugs have a variable ability to inhibit both forms of the cyclooxygenase enzyme, COX-1 and COX-2. NSAIDs, such as aspirin, indomethacin and ibuprofen, act by inhibiting both COX-1 and COX-2 nonselectivity, while currently a new drug called celecoxib acts as a selective inhibitor of COX-2 (Petersen and Nelson, 2010).

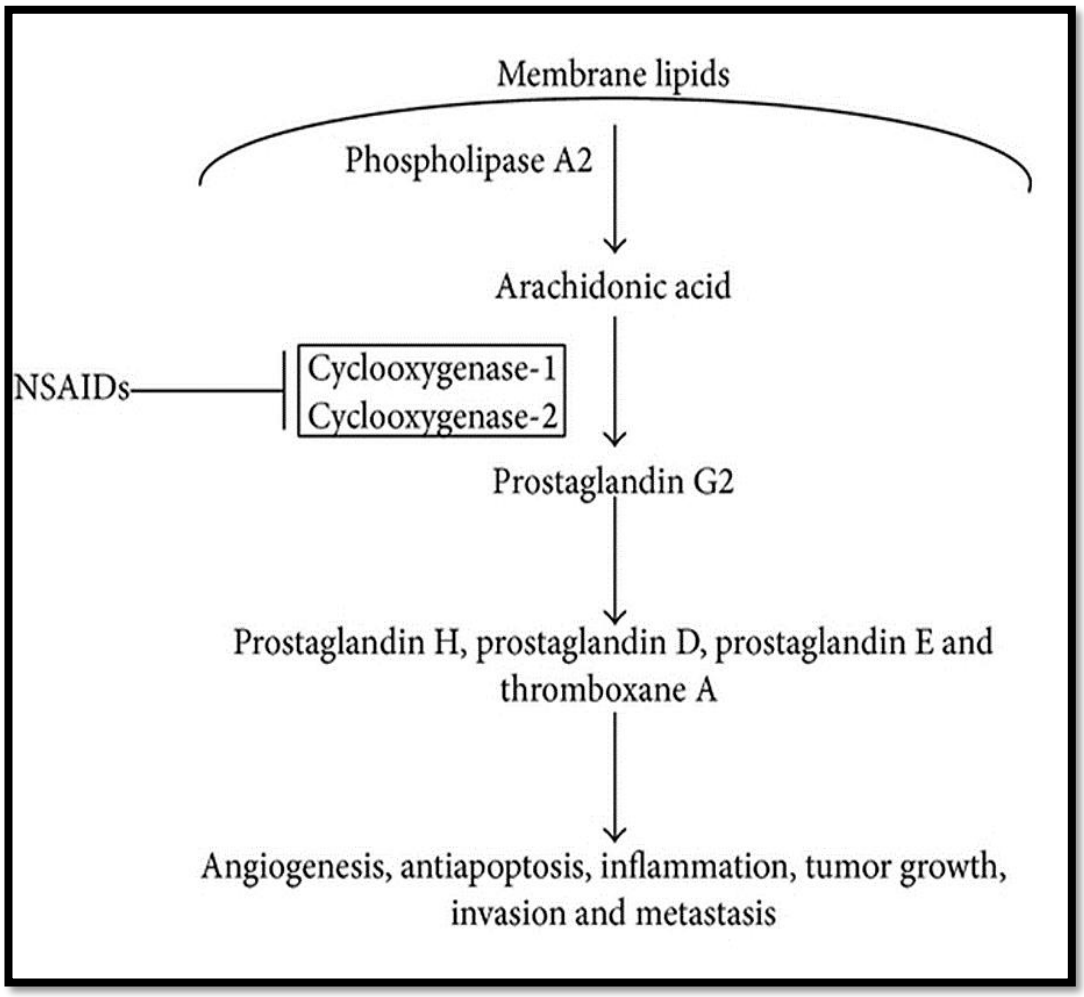



**Figure 2. The mechanism of action of NSAIDs**

NSAID inhibition of cyclooxygenase-1 and/or cyclooxygenase-2 suppresses prostaglandin G2 production, promoting apoptosis and blocking angiogenesis, inflammation, and tumour progression. Figure adapted from (Ishiguro and Kawahara, 2014).

### 1.8.4 NSAIDs and Cancer

The relationship between aspirin and antimetastatic effects was first determined when the link between platelet levels and metastasis was studied. It was observed that reduction of the platelet count inhibited the spread of malignant tumours, and consequently, a strong relation between the capacity of tumours to induce platelet aggregation and its capacity correlated with the metastasis was

discovered (Yan and Jurasz, 2016). Also, it has been observed that, in the circulation system, platelets were able to protect the tumour cells from the immune cells' system and promote their arrest on the endothelium, assisting the establishment of secondary lesions (Gay and Felding-Habermann, 2011). As a result of the relation between the thrombocytopenia with the decreased rate of metastasis, administration of the anti-platelet agent that inhibits platelet aggregation, NSAIDs, such as aspirin, were observed to significantly decrease the risk of distant metastasis (Karpatkin and Pearlstein, 1981; Rothwell et al., 2012), showing the ability of NSAIDs to control the development of cancer.

Prostaglandin (PG) can promote tumour growth, and therefore, abnormal PG synthesis is a characteristic of malignant cells. Thus, the advantage noticed with aspirin use was mediated through the inhibition of the COX enzymes that are in control of prostaglandin synthesis. Randomised trials for the prevention and reduction of colon adenomas demonstrated that aspirin can reduce the colon adenomas in patients with a prior history of adenoma, showing that using aspirin (600mg/day for ≥ 3 years) reduced the risk of colon adenomas after a 5-year latency period (Burn et al., 2012).

Further studies indicated that use of aspirin can reduce the rate of other cancers, including breast (Retsky et al., 2012), lung (Muscat et al., 2003), prostate (Kawahara et al., 2010), ovarian (Baandrup et al., 2015; Cramer et al., 1998), oesophagus, stomach, colon and rectum (Rose et al., 2011; Thun et al., 2002).

The expression of COX-2 has been found to be overexpressed in cancer tissue and associated with enhanced invasive, raised mutagenesis and proliferation mediated by Prostaglandin E2 (PGE2) and the production of reactive oxygen

species, and all these lead to an increased interest in NSAIDs for preventing and treating different kinds of cancers (Sobolewski et al., 2010).

In recent years, much attention has been paid to the consumption of selective COX-2 inhibitors; however, the relationship between the antitumour effects of platelet inactivation through COX-1 inhibition additionally added to the efficiency of NASIDs as tumour protective agents (Menter et al., 2014). Other anticancer effects include independent COX production by regulating the activation of the nuclear factor Kappa B (Stolfi et al., 2013).

## 1.9 Nanoparticles

The past decade has shown a huge development in nano biotechnology. Interest in nanoparticles (NPs) has increased in the past few decades, as they are effectively a bridge between the atomic or molecular structure and bulk material (Balavigneswaran et al., 2014). Nanoparticles are particles that vary in size from 10 nm to 1000 nm, and they can be presented in several forms, such as tubes, rods, wires or spheres, with more particular structures prepared, such as nano and nano peapods (Imasaka et al., 2006; Warner et al., 2008).

Nanoparticles have different physio-chemical properties from the bulk compound due to the small size of the particles. NPs have a great surface-area-to-volume ratio, which leads to an increased reactivity through higher contact between nanoparticles and the target tissue (Mu et al., 2014). Nanoscale offers the chance to make medicine with huge dissolution with other routes of administration and a more targeted delivery system, which can reduce therapeutic toxicity and extend the drugs circulating half-life, leading to reduced health care costs (Onoue et al., 2014).

However, this property is also believed to be responsible for negative biological effects. Therefore, in recent years, attention has increased regarding nanoparticles from a health and environmental safety point of view. The Royal Society and Royal Academy of Engineering (2004) produced the first report in this regard, focusing on the obvious shortage of information on how these engineered nanomaterials could affect human health and the environment. However, nowadays, there is no enough balance between the safety considerations and the growth of the material development of the nanotechnology industry. Moreover, the reason for the growing concern for nanoparticle toxicity is the increase in both the number and types of nanoparticles being encountered today (Kong et al., 2011).

### 1.9.1 Nanoparticles and cell interactions

NPs are capable of easily passing through cell membranes. Very small NPs (diameter < 4.5nm) can penetrate cell membranes by spontaneous penetration, while larger NPs (diameter > 4.5nm) can enter the cells via endocytosis and semi-endocytosis (Chen et al., 2013). These NPs may then be able to reach the nucleus through diffusion across the nuclear membrane or transportation through the nuclear pore and, therefore, allow direct activity on the nuclear DNA (Magdolenova et al., 2014). NPs can also functionalized with different molecules, such as antibodies, peptides and DNA/RNA to particularly target several cells (Sperling and Parak, 2010) and with biocompatible polymers (e.g., polyethylene glycol) to increase their *in vivo* circulation for drug and gene delivery applications (Ghosh et al., 2008; Nishiyama, 2007).

## 1.10 Methodology to Detect DNA Damage

### 1.10.1 Comet Assay

#### 1.10.1.1 Development of Comet assay

The Comet assay, also known as single cell gel electrophoresis (SCGE), is a sensitive technique for detecting DNA damage in cells (eukaryotic cells) induced by various types of genotoxicants *in situ* (Olive and Banath, 2006). This technique was first introduced by Ostling and Johanson in 1984 and, later in 1998, was modified by Singh and co-workers for use under alkaline conditions, which increased its reproducibility. The Comet assay has been known for its simplicity, sensitivity, time efficiency and cost effectiveness for assessing DNA integrity in the cells. It is considered to be a good indicator for measuring and detecting genotoxic damage (Gopalan et al., 2011).

#### 1.10.1.2 Principle of the Comet assay

The principle of this technique is simple; the procedure includes numerous steps. Firstly, nucleated cells are suspended in low melting point agarose and placed on Superfrost slides pre-coated with 1.0 % normal melting point agarose, the cells are then lysed with detergent and high salt concentration (Sodium chloride), to remove the cell membrane and proteins. DNA is allowed to unwind in alkaline buffer before being electrophoresed. The electrophoresis generates an electric field that forces the DNA fragments and single strands to migrate away from the nucleus toward the anode (Liao et al., 2009). Under alkaline conditions (pH>13), the double-stranded DNA is denatured and becomes single-stranded.

The slides are then neutralised with Tris buffer, pH 7.5. DNA is visualised with specific DNA binding dye, such as ethidium bromide (EtBr) or Sybr green in the dark and Comets are evaluated using a fluorescence microscope. The amount of DNA damage is then quantified within the head and the tail regions and measured together with the length of the tail. This allows for the analysis of an image that looks like a comet with a distinct head and tail. The head contains damaged DNA, while the tail consists of damaged DNA segments (Collins, 2004). Thus, the quantity of DNA found in the tail region would indicate the amount of damaged DNA in proportion to the undamaged DNA in the head region. The tail moment and the percentage of DNA in the tail are used as parameters to describe the DNA damage (Olive and Durand, 2005).

### 1.10.2 Comet repair assay

The Comet assay is an easy method for measuring DNA strand breaks in cells. The test is suitable for evaluating different chemicals for their genotoxicity, usage in human biomonitoring and epidemiology, and importantly, it also allows the measurement of DNA damage and repair (Azqueta et al., 2014). Controlling or monitoring the DNA repair is one of the crucial factors for monitoring cancer risk (Figueroa-González and Pérez-Plasencia, 2017). All cells have their own machinery for improving or repairing DNA damage before it becomes a permanent change in the DNA structure and leads to mutations (Torgovnick and Schumacher, 2015). The method of DNA repair is also called the challenge assay (Au et al., 2010).

#### 1.10.2.1 Principle of the Comet repair assay

Two main methods are available for assessing the repair of cellular DNA damage. The first method determines the repair of DNA damage following exposure of cells to a DNA damage inducing agent, and then measuring the damage after a short time interval (Azqueta et al., 2014). Generally, cells are exposed to chemicals such as hydrogen peroxide, bleomycin (BLM) or ionising radiation, and the repair percentage is assessed at chosen time intervals, which are usually short periods following exposure to the treatment. This is the commonest way of measuring lesions in most cell types. Further DNA damage, like UV-induced pyrimidine dimers using nucleotide excision repair and oxidised bases using base excision repair, can be checked by applying specific enzymes that can identify the lesions and convert these to strand breaks (Nikitaki et al., 2015). One enzyme, formamidopyrimidine DNA glycosylase (FPG), is used to convert 8-oxoguanine and other oxidised purines into single strand breaks (SSBs), whereas Alk A converts alkylated bases and T4 endonuclease V converts cyclobutane pyrimidine dimers (induced by UV) (Azqueta et al., 2011). Consequently, this assay allows monitoring of SSB–re-joining, BER by (tracking the removal of oxidised and alkylated bases) and NER by (monitoring the removal of UV-induced cyclobutane dimers) (Collins, 2014).

### 1.10.3 The micronucleus (MN) assay

Examination of DNA damage at the chromosome level is an important aspect of genetic toxicology. The micronucleus assay is an attractive tool for assessing chromosome damage as it allows missegregation of chromosomes and fragmented chromosomes to be measured reliably and there are OECD test guidelines (474, 487).

This type of damage can be observed as a micronucleus (MN) in cells during interphase. MNs are formed from a centric chromosomal fragment or entire chromosomes that lag behind at anaphase during nuclear division and are therefore rejected from the main nucleus (Fenech, 2007; Kirsch-Volders et al., 2011). The MN frequency could decline with repeated cell divisions and therefore a comparison between MN frequencies and dividing cell populations could not be determined (Fenech, 1997). During interphase, cytokinesis is blocked after treatment with cytochalasin B which is an inhibitor of the microfilament ring assembly, so producing binucleate cells that express MNs in mammalian cell cultures (Fenech, 2007).

#### 1.10.3.1 Advantages of the CBMN assay

The CBMN assay is used on several cell population types, including primary or transformed cell lines, such as mouse lymphoma cell lines and primary human lymphocytes. The CBMN assay uses general morphological criteria (Figure 3) that permit identification of cytotoxicity and genotoxicity by observing the following: chromosome breakage, chromosome loss, chromosome rearrangement (including nucleoplasmic bridges), cell division inhibition, apoptosis and necrosis (Fenech, 2007).

The assay also offers the alternative opportunity for chromosome aberration testing and the data obtained are not confounded by altered cell division kinetics caused by suboptimal cell culture conditions or cytotoxicity of the tested agents. Interphase cells can be assessed relatively objectively, which means the cells are much faster to score, so that scoring hundreds of cells per treatment group is possible and the resulting in data have substantial statistical power. Furthermore,

automated processes can be used to reduce scoring time even further (Doherty, 2012).

#### 1.10.3.2 Principle of the micronucleus assay

Micronuclei have been known for over 100 years in erythrocytes as the Howell-Jolly bodies seen on the examination of the blood. The first micronuclei were initially realised in *Vicia faba* (broad bean, fava bean) root tips exposed to X-rays (Evans et al., 1959), and this assay was used to study immature bone marrow erythrocytes in 1975. This test is regarded as sensitive, reliable and straightforward (Schmid, 1975). Micronuclei have been detected in erythrocytes using an *in vivo* assay; however, in recent studies, micronucleus detection has been achieved using an *in vitro/ex vivo* approach.

The micronucleus test is basically a mutagenic test system capable of identifying the cytotoxicity of certain chemicals. This cytotoxicity is demonstrated through the production of DNA fragments that are detached from the main nucleus and observable in the cytoplasm during interphase (Heddle et al., 2011). These pieces form from centric and acentric fragments of whole chromosomes that lack centromere regions. Accordingly, in anaphase of the cell-division cycle, these fragments are incapable of migrating in synchronisation with the other chromosomes, so they form micronuclei that are transferred to the daughter cells. Therefore, the development of micronuclei is a characteristic of chromosomal damage. Chemical exposure to cells that results in micronucleus formation would therefore indicate genotoxicity of that chemical and would be expected to affect the chromosome structure or modify segregation patterns (Fenech et al., 2011).

The numbers of micronuclei would indicate the genotoxic potential of a chemical (Hayashi, 2016).

#### 1.10.3.3 Cell culture for the micronucleus assay

The general procedure for producing micronuclei includes a 72 hour cell cycle that takes into account the cell culture period and the addition of phytohaemagglutinin (PHA) at 0 h. The stimulation is carried out by addition of the mitogen, cytochalasin B, at 44 h, followed by cell fixation at 72 h. After fixation of cells on slides and staining with a dye, the micronuclei are scored under a microscope.

Cell culture is imperative when working with the micronucleus assay, as micronuclei can only be obtained at interphase. The cells must undergo a nuclear division to show chromosome damage following *in vitro* exposure to a genotoxic agent. Cell cultures are initiated by the addition of lymphocytes (or other cells) to flasks of a medium containing PHA. The PHA stimulates cells by selectively stimulating T lymphocytes to enter mitotic cell division (Fenech et al., 2011). Cytochalasin B, a microfilament-assemble inhibitor is added to cells *in vitro* at 44 h to block cytokinesis, thereby causing the formation of bi-nucleated cells. At 72 h, the cells are subjected to a hypotonic shock and then fixed. to preserve the cytoplasm and cytoplasmic boundaries and to minimise clumping of the cells (Kirsch-Volders et al., 2011). The cells are dropped onto slides according to methods described by (Fenech, 2007) and then stained with Giemsa stain and scored under a microscope.

The addition of cytochalasin B, as described above, is imperative to halt cell division in the micronucleus assay. This particular method is known as the

cytokinesis block micronucleus (CBMN) assay. The bi-nucleate presence of cells confirms that the cells have divided following the presence of a test agent, thereby allowing the scoring of the micronuclei in those cells (Doherty, 2012).

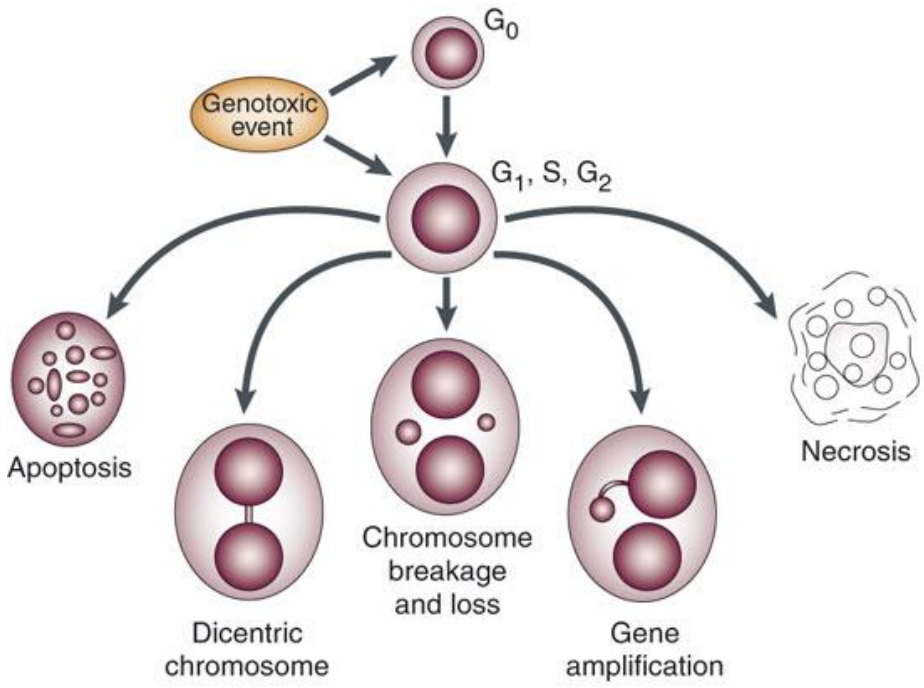



**Figure 3. Potential fates of cells after cell division**. The figure shows the different possible fates of cultured cytokinesis-blocked cells following exposure to cytotoxic/genotoxic agents. (Fenech, 2007).

#### 1.10.3.4 Types of micronuclei detected in the CBMN assay

Different kinds and forms of micronuclei can be produced. Categorisation first involves specification of the cells as mononucleated (containing one nucleus), binucleated (two nuclei) or multinucleated (more than two nuclei) (See Figure 4). These cell types may include one or several micronuclei. In addition, the CBMN

assay can measure nuclear buds (NBUDs), nucleoplasmic bridges (NPBs), and cell death (necrosis or apoptosis) (See Figure 4) as well as establish the nuclear division rate (Fenech et al., 2011). The presence of NBUDs indicates a lack of amplified DNA/DNA repair complexes, whereas nucleoplasmic bridges demonstrate DNA mis-repair and/or telomere end fusions (Luzhna et al., 2013).

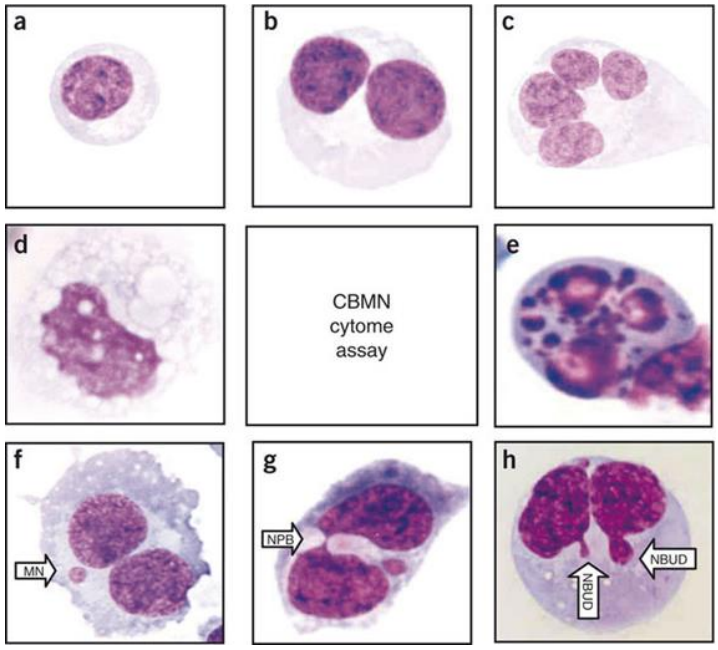



**Figure 4. Types of Micronuclei (MNi) in CBMN assay**. The figure shows Photomicrographs of several types of cells and biomarkers scored in the CBMN assay. **a**) Mononucleated cell; (**b**) BN cell; (**c**) multinucleated cell; (**d**) necrotic cell; (**e**) late apoptotic cell; (**f**) BN cell containing one or more MNi; (**g**) BN containing an NPB (and a MN); (**h**) BN cell containing NBUDs (Fenech, 2007).

## 1.11 Aims

The studies described in this thesis aim to assess the DNA damage and repair mechanisms induced by two well-known NSAIDs aspirin and ibuprofen in bulk and nano forms in human peripheral lymphocytes from prostate cancer patients versus healthy volunteers. To achieve this, first the genotoxicity of aspirin and ibuprofen were measured using the Comet assay. In addition, the effect of the drugs aspirin and ibuprofen in both forms on mitotic phases of the cell cycles was investigated by the micronucleus assay. Also, the protective effects of aspirin and ibuprofen in both forms were studied using the Comet challenge assay and ROS measurements. Furthermore, the p53 and XRCC3 protein expressions were investigated using Western blotting. Finally, the effects of anti-inflammatory drugs on major signal transduction pathways in isolated lymphocyte cells were studied by investigating the gene expression of ataxia-telangiectasia-mutated (ATM) kinase and ataxia-telangiectasia and Rad3-related (ATR) kinase in lymphocytes treated with aspirin and ibuprofen in bulk or nano forms were studied.

# Chapter 2

# Material and Methods

# 2 Material and Methods

## 2.1 Materials

The chemicals that were used in this work, as well as their sources and CAS numbers, are summarised in the following Table 1.2.

**Table 1.2 Chemicals with their corresponding sources and CAS numbers.**

| Chemicals | Supplier | CAS number |
|---|---|---|
| Aspirin | Sigma-Aldrich, UK | 50-78-2 |
| Acrylamide 30% | Sigma-Aldrich, UK | 79-06-1 |
| Anti-XRCC3 antibody | Abcam, UK | - |
| Ammonium persulphate APS | Sigma-Aldrich, UK | 7727-54-0 |
| Anti-Mutant p53 antibody | Abcam, UK | - |
| Anti-beta Actin antibody | Abcam, UK | - |
| Bovine serum albumin | Sigma-Aldrich, UK | 9048-46-8 |
| Bromophenol blue | Sigma-Aldrich, UK | 115-39-9 |
| Cytochalasin-B | Sigma-Aldrich, UK | 14930-96-2 |
| Disodium hydrogen phosphate (Na2HPO4) | VWR | 7558-79-4 |
| Dimethyl sulfoxide (DMSO) | BDH, UK | 67-68-5 |
| Donkey Anti-Rabbit IgG H&L (HRP) | Abcam,UK | - |
| Dithiothreitol (DTT) | Sigma-Aldrich, UK | 3483-12-3 |
| DPX Mountant | Sigma-Aldrich,UK | - |
| Ethanol | Sigma-Aldrich, UK | 64-17-5 |
| Ethidium bromide | Sigma-Aldrich, UK | 1239-45-8 |
| FAST SYBR, GREEN | Fisher Scientific, UK | 163795-75-3 |
| Formaldehyde | Sigma- Aldrich,UK | 50-00-0 |
| Foetal Bovine serum | Sigma-Aldrich, UK | N/A |
| Giemsa stain | VWR | 51811-82-6 |
| Glacial Acetic Acid | Fisher Scientific, UK | 64-19-7 |
| Glycerol | Sigma-Aldrich, UK | 56-81-5 |
| Hydrogen peroxide | Sigma-Aldrich, UK | 7722-84-1 |
| Ibuprofen | Albemarle sprt | 15687-27-1 |
| iScript™ cDNA Synthesis Kit | Bio-Rad,UK | - |
| Low melting point (LMP) agarose | Invitrogen, UK | 39346-81-1 |
| Methanol | Fisher Scientific, UK | 67-56-1 |
| Mitomycin C | Sigma-Aldrich, UK | 50-07-7 |
| $Na_2EDTA{\cdot}2H_2O$ | Sigma-Aldrich, UK | 6381-92-6 |
| NaCl | Sigma-Aldrich, UK | 7647-14-5 |
| NaOH | Fisher Scientific, UK | 1310-73-2 |

| | | |
|---|---|---|
| Normal melting point (NMP) agarose | Invitrogen, UK | 9012-36-6 |
| Penicillin-Streptomycin | Invitrogen, Ltd | - |
| phosphate buffered saline | Sigma, UK | N/A |
| Phytohemagglutinin | Invitrogen UK | 9008-97-3 |
| RPMI-1640 Medium | Sigma-Aldrich UK | N/A |
| Trizma Base | Sigma, UK | 77-86-1 |
| Trition X-100 | Sigma, UK | 9002-93-1 |
| Trypan blue | Sigma, UK | 72-57-1 |
| Potassium chloride | Sigma-Aldrich, UK | 7447-40-7 |
| Sodium phosphate diabasic | Sigma-Aldrich, UK | 7558-80-7 |
| Sodium phosphate monobasic | | 7558-80-7 |
| Western ECL Substrate | Bio-Rad,UK | - |
| Precision Plus Protein™ Dual | Bio-Rad,UK | - |
| Quick Start™ Bradford 1x Dye Reagent | Bio-Rad,UK | - |
| QIAamp RNA Blood Mini Kit | Qiagen, Germany | - |
| RNase | Sigma | - |
| 2-Mercaptoethanol | Sigma-Aldrich, UK | 60-24-2 |
| Protease inhibitor cocktail | Sigma-Aldrich, UK | 66701-25-5 |
| Supported nitrocellulose membrane 0.45µM | Bio-Rad,UK | 9004-70-0 |
| Trypan blue | Sigma-Aldrich, UK | 72-57-1 |
| Tween 20 | Sigma-Aldrich, UK | 9005-64-5 |
| Laemli sample buffer 2x | Sigma-Aldrich, UK | - |
| Quick Start Bovine Serum Albumin Standard | Bio-Rad, UK | 9048-46-8 |
| Hs_ACTB_1_SG QuantiTect Primer | Qiagen, Germany | - |
| Hs_ATM_1_SG QuantiTect Primer | Qiagen, Germany | - |
| Hs_ATR_1_SG QuantiTect Primer | Qiagen, Germany | - |

**Table 2.2 Equipment and other materials.**

| Equipments and other Services | Company / Distributor |
|---|---|
| Centrifuge Mistral 3000 | MSE, Albertville, USA |
| Centrifuge (biofuge 28 RS) | Heraeus, Sepatech, Germany |
| CCD camera | Hitachi KPMI/EK Monochrome, |
| Coplin jar | VWR, Lutterworth, UK |
| Cover slip | VWR, Lutterworth, UK |
| Culture flasks (25&75 $Cm^3$) | Corning Incorporated Costar®, |
| Dry incubator (37º C) LKB BIOCHROM | Leec LTD, Nottingham, UK |
| Electrophoresis power supply | Consort (E861), Belgium |
| Electrophoresis tank (HU20) | Scie-Plas, Renfrewshire, UK |
| Falcon tubes | BD, Swindon, UK |
| Freezer -20º C | Sanyo, Ultra low, Japan |
| Freezer -80º C | Sanyo, Ultra low, Japan |
| Fluorescent microscope | Leica, Weztler, Germany |
| Fume cupboard | Milton, UK |
| Fume hood ray air | Maiche Aire, Bolton, UK |
| Ice maker (Scotsman AF 100) | Namur, Belgium |
| Incubator 37º C with 5% $CO_2$ | Andor Technology Ltd, Belfast |
| Light microscope | Nikon, Japan |
| Komet 6 software | Kinetic Imaging, Nottingham, UK |
| Microcentrifuge MSE | GMI, Alberville, USA |
| Microplate reader | Dynex technology, Sussex, UK |
| Microscope (ortholux) | Leitz, Sturttgart, Germany |
| Mini protein 11 gel electrophoresis | Bio- Rad, Hertfordshire, UK |
| Pipettes | Gilson, Middleton, WI, USA |
| pH meter | Dunmow, UK |
| Power pack supply | Pharmacia LKB, Uppsala, Sweden |
| Super frost slides | VWR, Lutterworth, UK |
| Water bath | Grant instruments, Cambridge, UK |
| Zeta sizer-nano | Malvern instruments, UK |
| microplate reader | (Tecan, Switzerland). |

## 2.2 Methods

### 2.2.1 Ethical approval

The study was approved by the Ethics Committee of the University of Bradford, UK (reference number (0405/8), by Research Support & Governance Office, Bradford Teaching Hospitals reference number (Ref: RE DA 1202) and IRAS approval was obtained from Leeds East Research Ethics Committee Reference No, 12/YH/0464. All peripheral blood samples were collected after the patients signed informed consent

#### 2.2.1.1 Blood collections

Whole blood samples were collected by venipuncture from 20 healthy male volunteers and 20 prostate cancer patients (see tables 2 and 3) after signing a consent form (Appendix I); a questionnaire (Appendix II) was completed prior to sample collection and information sheet provided (Appendix III). Samples were diluted at the rate of 1:1 with RPMI-1640 medium (Sigma Aldrich) supplemented with 10% dimethyl sulfoxide (DMSO). The mixture was transferred into labelled Eppendorf® tubes and was immediately frozen in the -80°C freezer.

**Table 2.3 Brief information about the samples used from healthy donors.**

| No | Sample code | Age | Ethnicity | Gender | Smoking history | Family history |
|---|---|---|---|---|---|---|
| 1 | NG | 47 | Asian | Male | Smoker | - |
| 2 | M.26-9.14 | 42 | Arab | Male | Smoker | - |
| 3 | Y.23.10.14 | 55 | Arab | Male | Non-smoker | - |
| 4 | A6.10-24 | 45 | Arab | Male | Smoker | - |
| 5 | W23-09-14 | 47 | Arab | Male | Non-smoker | - |
| 6 | J12-6-13 | 35 | Asian | Male | Non-smoker | - |
| 7 | F26-9-14 | 56 | Arab | Male | Non-smoker | - |
| 8 | P.18-6-13 | 37 | Asian | Male | Non-smoker | - |
| 9 | AA | 26 | Asian | Male | Non-smoker | - |
| 10 | O-12-6-13 | 40 | Asian | Male | Non-smoker | - |
| 11 | M5-12 | 50 | Caucasian | Male | Smoker | - |
| 12 | NA | 34 | Asian | Male | Smoker | - |
| 13 | M5.12.14 | 42 | Asian | Male | Smoker | - |
| 14 | J15-1.15 | 34 | Arab | Male | Non-smoker | - |
| 15 | 001PAG 06.09.17 | 40 | Asian | Male | Non-smoker | - |
| 16 | S16.12.14 | 38 | Asian | Male | Smoker | - |
| 17 | SH17.01.17 | 56 | Asian | Male | Smoker | - |
| 18 | K12-05-15 | 66 | Caucasian | Male | Smoker | - |
| 19 | M23-01-15 | 63 | Caucasian | Male | Non-smoker | - |
| 20 | A12-06-15 | 48 | Caucasian | Male | Non-smoker | - |

**Table 2.4. Brief information about the prostate cancer patients.**

| N0 | Sample code | Age | Ethnicity | Gender | Smoking history | Family history |
|---|---|---|---|---|---|---|
| 1 | 4239 PSC | 61 | Caucasian | M | Smoker | - |
| 2 | 3497 PSC | 80 | Asian | M | Past smoker | - |
| 3 | 3498 PSC | 64 | Caucasian | M | Past smoker | - |
| 4 | 3511PSC | 82 | Caucasian | M | Past smoker | - |
| 5 | 3514 PSC | 65 | Caucasian | M | Smoker | - |
| 6 | 3515 PSC | 73 | Caucasian | M | Past smoker | - |
| 7 | 4232 PSC | 63 | Asian | M | Non-smoker | - |
| 8 | 4233 PSC | 67 | Caucasian | M | Non-smoker | - |
| 9 | 4234 PSC | 55 | Caucasian | M | Smoker | - |
| 10 | 4240 PSC | 67 | Caucasian | M | Past Smoker | - |
| 11 | 4244 PSC | 66 | Asian | M | Past smoker | - |
| 12 | 4245 PSC | 79 | Caucasian | M | Past smoker | - |
| 13 | 4249 PSC | 76 | Caucasian | M | Past smoker | - |
| 14 | 5333 PSC | 60 | Caucasian | M | Non-smoker | - |
| 15 | 5376 PSC | 68 | Caucasian | M | - | - |
| 16 | 3499 PSC | 72 | Caucasian | M | Smoker | - |
| 17 | 5178 PSC | 72 | Caucasian | M | Past smoker | - |
| 18 | 4231 PSC | 83 | Caucasian | M | Past smoker | - |
| 19 | 5371 PSC | 77 | Caucasian | M | Past smoker | - |
| 20 | 5374PSC | 60 | Asian | M | Smoker | - |

### 2.2.2 Preparation of milled nano-suspensions and quality control

Suspensions of both aspirin and ibuprofen were made at solids loads of 5% (w/w) and 4% (w/w) respectively in a medium consisting of hydroxypropyl methylcellulose (HPMC) (0.5%, w/w), polyvinylpyrrolidone K-30 (0.5%, w/w) and sodium lauryl sulphate (0.1%, w/w). The 5% (w/w) aspirin and 4% (w/w) ibuprofen solutions were processed in deionised water at neutral pH. A Lena Nanoceutics Technology DM-100 machine was used for the milling process. The milling of 250ml of each suspension was performed for 60mins with 150ml of 0.2mm yttrium stabilised zirconium beads (Glen Mills, USA). The suspensions were transferred to an impervious glass bottle and stored in the refrigerator at 4°C for the duration of the experiments.

### 2.2.3 Zeta potential

The mean particle sizes in the stock solutions of both aspirin and ibuprofen were measured by Photon Correlation Spectroscopy using a Zetasizer Nano ZS-90 Model ZEN3600 (Malvern Instruments Ltd, UK). All measurements were carried out at room temperature (RT) using disposable sizing cuvettes.

### 2.2.4 Particle morphology and visualisation

To examine the particle surface morphology of the aspirin and ibuprofen nano-particles, a scanning electron microscope was used (FEI Quanta 400, Cambridge, UK). Both samples were placed on a graphics layer on an aluminium stub with a conductive double-sided carbon tape. The photographs were taken at a variety of magnifications operated at 10kV. To visualise the inherent matrix of individual particles and their shape, transmission electron microscopy TEM Tecnai 12 (FEI

Company, Netherlands) was used. Aspirin and ibuprofen suspensions were diluted to 1ml with purified water, and then 7µl of diluted samples were mounted on carbon-coated copper TEM grids allowing thorough air-drying before viewing.

### 2.2.5 Stability of the aspirin and ibuprofen nano-suspensions

Aspirin and ibuprofen nano-suspensions were checked each month for any sedimentation and changes in particle size to determine any agglomeration or aggregation. The particle size was measured using the Zetasizer Nano.

### 2.2.6 Aspirin and ibuprofen doses

In this study, two different forms of aspirin and ibuprofen were used (NPs and bulk). In both forms, the same concentration 500µg/ml was used for the Comet assay, while 200µg/ml were used for ibuprofen nano and bulk, and 500µg/ml of aspirin nano and bulk were used for the micronucleus assay.

### 2.2.7 Comet assay

#### 2.2.7.1 Cell treatment

A volume of 890µl of RPMI medium was used as a suspension and 10µl of treatment solutions (aspirin, ibuprofen at 500 µg/ml and Hydrogen peroxide ($H_2O_2$) at 50 µM) , and 100µl of whole blood was added in Eppendorf® tubes with a final volume of 1,000µl and then incubated for 30mins at 37°C. The tubes were centrifuged at 3000 rpm for 5mins, and then 900µl of the supernatant was discarded. The cell pellet was re-suspended and mixed in 100µl of 0.5% low melting point (LMP) agarose prepared with PBS; then 100µl of cell suspensions were placed on a dry slide pre-coated with 1% of normal melting point agarose (NMP). An appropriately sized coverslip was employed quickly to spread out each

agarose layer; the agarose was allowed to set on ice for 5mins.

#### 2.2.7.2 Cell lysis

Once the slide held two agarose layers, the coverslip was then removed, and the slide was immersed in freshly prepared cold lysis solution, a high salt solution containing a detergent (100mM EDTA, 2.5M NaCl, 10mM Trizma base, 10% DMSO and 1% Triton X-100, at pH 10) for at least one hour or overnight**.**

#### 2.2.7.3 Electrophoresis

Before electrophoresis, the slides were placed in a horizontal gel electrophoresis tank and equilibrated in fresh cold alkaline electrophoresis solution (10M NaOH and 200mM EDTA, pH >13). The slides were kept in an electrophoresis tank for 30mins at 4°C. Electrophoresis was conducted at 4°C for 30mins using constant voltage at about 25 volts and 300mA current.

#### 2.2.7.4 Neutralisation

After electrophoresis, the slides were removed from the electrophoresis solution and a neutralising buffer (0.4M Trizma base, pH 7.5) was applied three times for 5mins. The DNA was visualised by staining the slides with 60µl of (20µg/ml) ethidium bromide and the slides were covered with a coverslip.

#### 2.2.7.5 Scoring of slides

All slides were coded before scoring, and 100 cells were scored double blind per observation using a fluorescence microscope equipped with a CCD camera and computer system. Data were generated measuring the Olive tail moment and % Tail DNA using Komet 6 software, Kinetic imaging (Andor Technology Ltd, Belfast, UK).

#### 2.2.7.6 Statistical analysis

Mean data were generated with standard errors. The data obtained were tested for normality of distribution using the Kolmogorov-Smirnov test. Data were analysed using one-way analysis with post hoc test to determine significance relative to control. In all cases, P was considered significant at $P < 0.05$. All analyses were performed using SPSS for Windows statistical package (version 22).

### 2.2.8 Micronucleus assay

Fresh blood samples were obtained from five healthy men and five prostate cancer patients. Samples were used for cell culture after collection in heparinised tubes.

#### 2.2.8.1 Cell culture and treatment

All culture steps were carried out under sterile conditions and any chemicals were added to the culture flasks using a fume hood. 4.5ml of the basic culture medium (RPMI-1640 containing 25mM HEPES and L-Glutamine, 15% foetal bovine serum, 1% penicillin-streptomycin solution) was transferred into each $25cm^3$ Corning® vented cap culture flasks and stored at -20°C until ready for use. The flasks were equilibrated in a 37°C incubator (5% $CO_2$) at least 30mins before use.

#### 2.2.8.2 Culture started

At 0hrs, 130µl of phytohaemagglutinin (PHA) and 300µl of fresh blood were added to each flask. The culture flasks were mixed gently and then incubated at 37°C for 24hrs in the presence of 5% $CO_2$.

At 24hrs, 50µl of each chemical was added, 50µl of RPMI-1640 was added to the negative control (NC) cultures. 0.4 µM of mitomycin C was used as a positive control (PC) and ASP B, ASP N were added at 500 µg/ml, IBU B and IB N at 200 µg/ml. The culture flasks were then incubated for an additional 20hrs.

At 44 h, 30µl of 1mg/ml of cytochalasin-B (cyto-B) was added to each culture to halt cytokinesis. The flasks were then incubated for an additional 28hrs.

At 72hrs, flasks were removed from incubation and then the contents transferred into 15ml Falcon® tubes. At this time sterile conditions were no longer required. The 15ml Falcon® tubes were centrifuged for 8 min and elsewhere at 800 rpm. Then, for each culture, the supernatant was removed using a vacuum pump until 500µl remained. To allow the hypotonic shock to take effect on the cells, 5ml of cold (4°C) 90mM KCl was added to each tube while being gently mixed on a vortex followed by a 15mins incubation at 4°C. Tubes were centrifuged, and a vacuum pump was used to discard the supernatant with retention of 500µl each.

#### 2.2.8.3 Fixation of cells

Five ml of freshly prepared Carnoy's solution (one part glacial acetic acid and three parts methanol; the solution was always freshly prepared) was added drop by drop while being gently mixed on a vortex followed by three drops of 38% formaldehyde to each tube mixed with a Pasteur pipette (Fisher Scientific). The cell suspensions were centrifuged at 800 rpm for 8mins, and the supernatants were discarded using a vacuum pump, retaining approximately 500µl each. The fixation process was repeated twice without the addition of formaldehyde. The tubes were left overnight at 4°C.

### 2.2.8.4 Slide preparation, staining and mounting slides

The following day, the cell solutions were centrifuged for eight minutes and the supernatant discarded until approximately 100µl remained. Then, depending on cell density and pellet size, between 200-600µl of fresh Carnoy's solution was added. A volume of 20µl of cell suspensions was dropped twice onto a labelled frosted glass slide (centre left and centre right) and left to air-dry. Four slides were used for each treatment group, and the cell density was checked using a phase-contrast microscope (Nikon Eclipse Ti-S) before being left to dry at room temperature overnight. The slides were stained for 20mins in a Giemsa in Sorenson buffer (5% Giemsa solution in phosphate buffer at pH 6.8). Slides were gently washed for approximately 2mins and left to air-dry. Coverslips 24 × 50mm$^2$ (VWR) were mounted onto the prepared slides using three drops of DPX Mountant on heating block at 40°C and left to set overnight. The slides were viewed under a light microscope (Nikon Eclipse E-200).

### 2.2.8.5 Micronuclei (MN) scoring

For determining the MN frequency detected with the assay, 1,000 cells for each dose point were scored under 40x magnification using a light microscope. Scoring was done according to the criteria described by Fenech (2007) (Figure 1.2). The frequency of MN in 1,000 binucleated (BiNC) lymphocytes was used to calculate the nuclear division index (NDI); M1-M3 representing the number of cells containing 1-3 nuclei and N is the total number of viable cells scored.

**NDI = (M1 + 2(M2) + 3(M3)) /N**

M1 = mononucleated cells, M2 = binucleated cells, M3 = multinucleated cells, N = the total number of viable cells scored.

The number of cells scored for the investigation was 1,000 cells/person/treatment group. The NDI provides a measure of the mitogen-induced cell cycle of viable cells and the effect of the compound on the cell cycle. The NDI values frequently range from 1.0 to 2.0. However, if all the viable cells have been able to complete more than one nuclear division during the cytokinesis-block phase with the number of mononucleated cells (MoNC) having decreased and the number of multinucleated cells (MultiNC) greatly increased, the NDI value may even be greater than 2.0.

## BOX 1 | SCORING CRITERIA

### Criteria for scoring viable mono-, bi- and multinucleated cells

Frequency of viable mono-, bi- and multinucleated cells is measured to determine cytostatic effects and the rate of mitotic division, which can be calculated using the nuclear division index (see **Box 3**). These cell types have the following characteristics:

- Mono-, bi and multinucleated cells are viable cells with an intact cytoplasm and normal nucleus morphology containing one, two and three or more nuclei, respectively.
- They may or may not contain one or more MNi or NBUDs and in the case of bi- and multinucleated cells they may or may not contain one or more NPBs.

Necrotic and apoptotic cells should not be included among the viable cells scored.

On rare occasions, multinucleated cells with more than four nuclei are observed if cell-cycle time is much shorter than normal or the cytokinesis-blocking time is too long.

### Criteria for selecting BN cells suitable for scoring MNi, NPBs and NBUDs

The cytokinesis-blocked BN cells that may be scored for MN, NPB and NBUD frequency should have the following characteristics:

- The cells should be binucleated.
- The two nuclei in a binucleated cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary.
- The two nuclei in a binucleated cell should be approximately equal in size, staining pattern and staining intensity.
- The two nuclei within a BN cell may be attached by a nucleoplasmic bridge, which is no wider than 1/4th of the nuclear diameter.
- The two main nuclei in a BN cell may touch but ideally should not overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of each nucleus are distinguishable.
- The cytoplasmic boundary or membrane of a binucleated cell should be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells.

### Criteria for scoring micronuclei

MNi are morphologically identical to but smaller than nuclei. They also have the following characteristics:

- The diameter of MNi in human lymphocytes usually varies between 1/16th and 1/3rd of the mean diameter of the main nuclei, which corresponds to 1/256th and 1/9th of the area of one of the main nuclei in a BN cell, respectively.
- MNi are non-refractile and they can therefore be readily distinguished from artifact such as staining particles.
- MNi are not linked or connected to the main nuclei.
- MNi may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.
- MNi usually have the same staining intensity as the main nuclei but occasionally staining may be more intense.

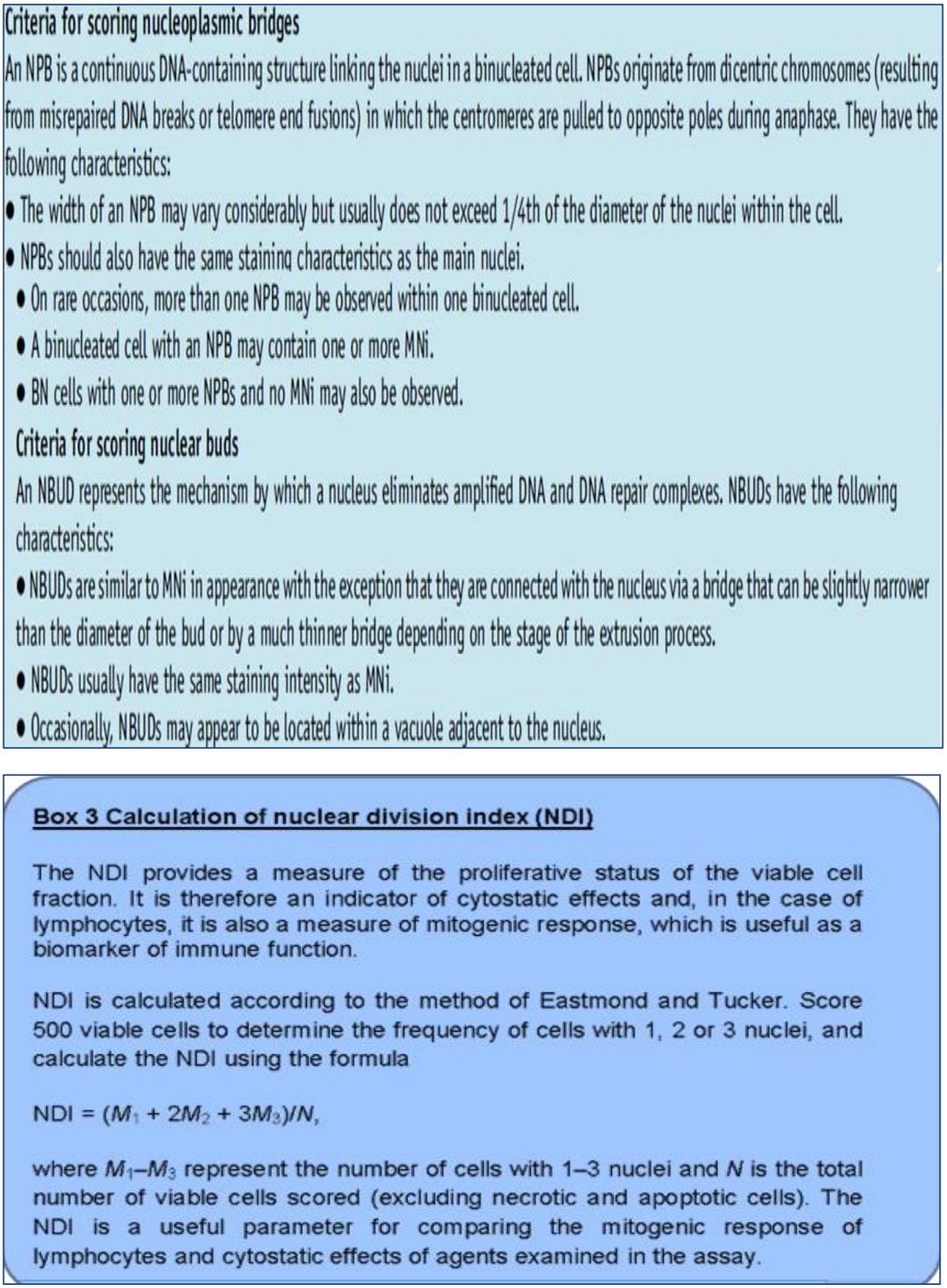



**Figure 2.1 MN scoring criteria adapted from Fenech (2007) and Eastmond and Tucker(1989).**

#### 2.2.8.6 Statistical analysis

Each experiment was done five times independently. All values presented are represented as mean values ± standard deviations (SD). To determine the difference between the different treatment groups, a normality test was performed. One-way ANOVA and an independent t-test were used to determine association and comparisons between independent groups using SPSS 22.0 for Windows (SPSS Inc.).

### 2.2.9 Methods for Western blot

#### 2.2.9.1 Human lymphocyte isolation

Fresh peripheral blood from prostate cancer patients and healthy individuals were used for isolating the lymphocytes**.** The whole blood was transferred to a Falcon™ tube and diluted in 1:1 with 0.9% saline. Six ml of this dilution was carefully loaded on the 3ml lymphoprep layer, followed by centrifugation for 20mins at 1,900rpm without disturbing the lymphoprep layer. The middle white buffy coat layer, containing the lymphocytes, was transferred to tubes prefilled with 10ml of normal saline for another washing and centrifuged at 1,500rpm for 15mins at RT. The supernatant was carefully discarded, and the pellet was re-suspended in 900µl of RPMI-1640 medium.

#### 2.2.9.2 Determination of the cell concentration and density

After the isolation, the concentration of the lymphocytes was determined by counting the cells using a haemocytometer. Fifty µl of 0.4 trypan blue solution (Sigma, UK) was added to 350µl of RPMI medium, and 100µl of cell stock suspension was added to a 1.5ml Eppendorf® tube and mixed vigorously by vortexing. Ten µl of cells/trypan mixture was transferred to each chamber of the

haemocytometer by pipetting very gently both chambers underneath the coverslip and cells were allowed to settle over the grid. The total number of the lymphocyte cells was counted in the four squares of the corner of each chamber under the light microscope using the 10x objective lens and focusing on the grid lines of the chamber.

The lymphocyte cells concentration was calculated from the following formula:

Cells/ml = Average number of cells in one large square × dilution factor* × $10^4$**

Dilution factor was 5 (1 volume cell suspension: 4 volume of diluted trypan blue)

**$10^4$ is the conversion factor to convert $10^{-4}$ml (volume of one large square) to 1ml.

$$Measured\ cell\ density = \frac{Average\ cells\ per\ small\ square \cdot Dilution\ factor}{Volume\ of\ a\ small\ square\ (mL)}$$

### 2.2.9.3 Exposure of lymphocyte cells to the chemicals

The lymphocyte cells were seeded at a concentration of 1 x $10^6$ cells in six well plates and cultured for 24hrs in complete RPMI-1640 medium containing 15% fetal bovine serum (FBS) (Invitrogen Ltd, Paisley, UK) and 1% penicillin-streptomycin from Gibco, Paisley, UK at normoxic condition (37°C, 5% $CO_2$ and 100% humidity). After 24hrs' incubation, RPMI media was replaced with fresh basic RPMI medium and cells were treated with 500µg/ml aspirin bulk, nano-size and 200µg/ml both forms of ibuprofen respectively and the negative control was cultured without treatment. Cells were incubated for another 24hrs.

#### 2.2.9.4 Protein extraction and sample preparation

After 24hrs of treatment, the medium was discarded, and the lymphocytes washed twice with 2ml of cold PBS. Then the cultured cells were lysed in 150µl of lysis buffer (0.048M Tris-HCl, pH 6.8 containing 0.8mM sodium pyrophosphate, 5mM EDTA, 2% w/v sodium dodecyl sulphate (SDS), 10% glycerol, 50 mM dithiothreitol (DTT), 0.01% w/v bromophenol blue). A cell scraper was used to harvest the cells and homogenised by passing the lysate through a 23G gauge needle several times. Next, samples were transferred to new labelled microcentrifuge tubes and then stored at -20°C until analysis.

#### 2.2.9.5 Determination of protein concentration and sample preparation

The protein concentration was qualified using a Bio-Rad Bradford assay kit following the manufacturer's instructions. This assay is based on colourimetric changes caused by an absorbance shifting in the dye coomassie (red colour) into coomassie blue by binding to the protein. Five serial dilutions (0.125mg/ml to 2mg/ml) of Bovine Serum Albumin (BSA) were used as standers, and distilled water was used as a blank. Five µl of each unknown sample, standard and distilled water, were pipetted into separate wells of 96 well plates (Sigma, UK) in triplicate, and 250µl of diluted Bio-Rad dye reagent (Bio-Rad, Hertfordshire, UK) added to each well. The plate was incubated at room temperature for 5mins and absorbance measured at wavelength 595nm on a microplate reader (Tecan, Switzerland). The results of the absorbance values for unknown samples were used to produce a standard curve, and the protein concentrations of the samples were calculated.

### 2.2.9.6 Polyacrylamide gel preparation

The gel loading assembly (Bio-Rad) was assembled following the manufacturer's instructions. Tris buffers at pH 6.8 and pH 8.8 were prepared for the resolving (1.5M Tris and 0.4% w/v SDS, pH 8.8) and stacking gels (0.5M Tris and 0.4 w/v SDS, pH 6.8), then stored at room temperature. A 12% resolving gel was prepared and poured directly into the assembled apparatus (Appendix V) and the mixture overlaid with 0.5ml of 0.1% (w/v) SDS. The gel was allowed to set at RT for one hour. After pouring the SDS off, a 5% stacking gel was prepared with pH 6.8 and then added on the top of the solidified resolving gel (Appendix V). A gel comb was inserted, and the gel was allowed to sit at room temperature for about 30mins. After that, the comb was then removed, and the gel apparatus was transferred to an electrophoresis buffer tank (Bio-Rad) filled with 1 x running buffer (25mM Tris base, 192mM glycine and 0.1% w/v SDS, pH 8.3). While gels were setting, the protein samples were prepared by re-suspending in 4x laemmlis loading buffer and denaturised at 95ºC for 7mins; the samples were left to cool down at RT. Once the gel set, the gel apparatus was placed in an electrophoresis buffer tank (Bio-Rad) containing 1 x running buffer. Thirty µg/ml of protein samples were loaded into the separate gel and 9µl pre-stained protein ladder (Bio-Rad, UK). The polyacrylamide gel was run at 50 volts for 30mins, then run at 100 volts for 1hr 30mins.

### 2.2.9.7 Protein transfer to membrane

After the electrophoresis, a Bio-Rad mini transfer kit was used for transferring the proteins to a nitrocellulose membrane under wet blotting condition. A transfer was prepared by placing the gel against a nitrocellulose membrane (Bio-Rad, UK) and sandwiched with filter papers and sponges. A transfer tank was filled

with blotting buffer (25mM Tris base, 192mM glycine and 20% v/v methanol) and run at 100 volts for about 2hrs.

#### 2.2.9.8 Detection of the protein

Once the transfer was completed, the membrane was immersed in 25ml of blocking buffer of 5% w/v Bovine Serum Albumin (BSA) in Tris buffer saline containing Tween 20 (TBS-T) (150mM sodium chloride, 20mM Tris base, 0.1% (v/v) Tween 20, pH 7.4) on a shaker for one hour at room temperature to block nonspecific site binding sites. The blocking solution was poured off, and the membrane was then incubated with the p53 mouse (1:2,000) or XRCC3 (1:500) or P21 (1:2000) (Table 2.1) at 4ºC on a shaker overnight. The following day, the membrane was washed with TBST (3 × 5mins) and afterwards incubated with 20ml of 5% blocking solution containing either horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG (1:10,000) or horseradish peroxidase (HRP)-conjugated sheep anti-rabbit IgG (1:10,000) for 1hr at room temperature depending on which primary Ab was used on shaker at RT. Finally, the membrane was washed with TBST (3 × 5mins) and then prepared for the detection of the bands.

#### 2.2.9.9 Enhanced chemiluminescent (ECL) detection

After the final wash, the membrane was enhanced by incubating the membrane with the same amount of ECL solution 1 and 2 for 1min at room temperature. Excess of detection reagent was removed, and the membrane was placed on GB Box (Gene flow, UK). The same membrane was washed with TBST (3 × 5mins) and re-blotted with primary and secondary antibodies to detect actin protein (Table 2.1).

#### 2.2.9.10 Data analysis

All western blotting experiments were carried out with at least three independent samples. Image J software was used to analyse the band intensity. The expression level of a protein of interest was normalised to housekeeping proteins (ß-Actin) of the same sample. The fold change in the expression of the target proteins upon treatment was calculated and the expression level in treated samples was normalised to untreated controls.

### 2.2.10 Methods for qPCR

#### 2.2.10.1 RNA extraction and quantification

Total RNA was extracted from the whole blood samples obtained from healthy individuals and prostate cancer patients exposed to both forms of aspirin and ibuprofen using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Briefly, 2ml of whole human blood was mixed with 10ml of Buffer EL in a 15ml Falcon™ tube and then incubated in ice for 15mins with mixing two times during the incubation. The mixer was centrifuged at 800rpm for 10mins at room temperature and then completely removed and discarded supernatant. The pellet cells were suspended in 8ml of EL buffer by vortexing briefly, then the cells were pelleted by centrifuging for 10mins at 800rpm. EL buffer was discarded and removed. Cells pellets were disrupted in 350µl of the Buffer RLT and homogenised by centrifuging for 2mins at maximum speed using a QIAshredder spin column in a 2ml collection tube. About 600µl of 70% ethanol was added to the homogenised lysate and mixed well by pipetting. The sample was pipetted, including any precipitate which may have formed, into a new QIAamp spin column placed in a 2ml collection tube (QIAGEN) and

centrifuged for 15s at 10,000rpm. The QIAamp spin column was washed once with 350µl Buffer RW1 in advance performance on-column DNase digestion with the RNase-Free DNase set (QIAGEN). Briefly, DNase I incubation mix was prepared from DNase I stock solution according to the manufacturer's instructions. Eighty µl of DNase I incubation mix was added directly to QIAamp spin column membrane and placed on the bench for about 25mins. Following this, the spin column membrane was washed with 350µl of W1 Buffer and centrifuged for 15s at 10,000rpm. The spin column was washed twice with RPE Buffer then centrifuged at 14,000rpm for 3mins to avoid carrying over of ethanol. Finally, the QIAamp spin column was transferred to a new 1.5ml microcentrifuge tube, and RNA was eluted by pipetting 50µl of RNase-free water directly onto the QIAamp membrane followed by centrifugation for 1min at 10,000rpm. The purity and quality of RNA were measured using a NanoDrop™ 1000 spectrophotometer that measures the absorbance of UV light and calculates the 260/280 ratio (Thermo Scientific). Finally, RNA samples were used for cDNA synthesis and stored at -80ºC until being used for qPCR.

#### 2.2.10.2 Complementary DNA synthesis

To synthesise single-stranded cDNA from total RNA the iScript cDNA Synthesis Kit (Bio-Rad) in 20µl reaction volumes was used by mixing the following component in order: 4µl of 5x iScript Reaction Kit, RNase-free water, and one µg of specimen RNA. PCR reactions were performed in Bio-Rad PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories, Inc. Hercules, CA, USA), using the amplification conditions listed in (Table 2.5) below.

| Step | Time | Temperature |
|---|---|---|
| **Incubation/ primer annealing** | 5 min | 25ºC |
| **cDNA synthesis** | 15 min | 42ºC |
| **Enzyme inactivation** | 5 min | 95ºC |

**Table 2.5 the reaction protocol of cDNA synthesis.**

### 2.2.10.3 QRT-PCR method

After synthesising the cDNA, the real-time PCR reaction was set up in 96 well plates (MicroAmp$^{TM}$) for each gene of interest to be quantified in triplicate to reduce variation in a UV-irradiated hood on 7500 RT-PCR System (Applied Biosystems).

Real-time PCR reaction was mixed for 20µl volume reactions consisting of 2µl of primer for the target gene ATM, ATR and β-Actin (Qiagen, UK), 10µl of SYBR™ Green PCR Master Mix Brands (ThermoFisher Scientific, UK), 4µl of RNase/DNase-free water (Qiagen, UK) and 4µl of diluted cDNA. The endogenous control (β-Actin) was used as a housekeeping gene for the normalisation of the reaction. The qRT-PCR thermal conditions were set up as follows. Firstly, denaturation at 95°C for 10mins then 40 cycles of denaturation at 95°C for 15s, annealing and extension at 60°C for 1min. Finally, a melting curve of 95°C for 15s, 60°C for 15s and 95°C for 15s.

### 2.2.10.4 Data analysis

Real-time PCR assay selected in this experiment is based on the detection of the fluorescence by using fluorescence reporter molecules such as SYBR Green that includes dyes that bind to double strand DNA. The increase in the

fluorescence intensity is proportional to the accumulation of PCR product with each cycle of amplification, with qPCR instrument systems (7500 Applied Biosystems) and then collecting the data for each sample during each PCR cycle.

The early cycle at which the amplification formed fluorescence can be detected mainly as setting slightly above the ambient background signal is called the threshold cycle 'CT'. The numerical value of the CT is represented as the amount of genes of interest in the reaction (i.e. the lower the CT level, the greater the amount of target) (Schmittgen and Livak, 2008).

The quantification analysis was employed to quantify the expression of the target gene and then compared with the normalising gene β-Actin. The ΔCT method was processed to determine the relative expression of the target gene.

This method is a convenient way to calculate CT values and inversely correlated gene expression by directly using the threshold cycles (CTs).

The lowest ΔCT value is the highest expression). The ΔCT values were calculated using the following:

ΔCT (sample) = CT gene of interest - CT internal control gene

The 2-ΔΔCT method was used to compare the fold change of gene expression between untreated lymphocytes (control cells) from prostate cancer patients and healthy individuals, and lymphocytes treated with both forms of aspirin and ibuprofen.

The ΔCT value was firstly calculated for each sample using the following equation:

ΔCT (sample) = CT target gene - CT housekeeping gene

ΔCT (untreated cells) = CT target gene - CT housekeeping gene. Subsequently, the ΔΔCT value for each sample was calculated using the following equation:

ΔΔCT = ΔCT (sample) - ΔCT (control cells).

Finally, the ΔΔCT formula was used to estimate the fold change values between untreated and treated lymphocytes (fold change = $2^{-\Delta\Delta CT}$).

#### 2.2.10.5 Statistical analysis

The significance was calculated by using a two-tailed student t-test. The result was expressed as the mean ± standard deviation. P value was calculated to determine the statistical significance of the results.

### 2.2.11 Bleomycin Challenge assay

A modification of the basic alkaline comet assay was introduced to measure DNA repair capacity in human lymphocytes from healthy individuals and prostate cancer patients. Treatment groups for all samples have been described in this section. Scoring of cells and statistical analysis has been described in sections 2.2.7.5, 2.2.7.6.

#### 2.2.11.1 Experiential design

This experiment was performed to evaluate the baseline and DNA repair capacity of lymphocytes, after bleomycin exposure to lymphocytes from healthy control individuals and prostate cancer patients. In addition to the baseline Comet assay described before in section 2.2.6, lymphocytes were treated with bleomycin at concentration; 5µg/ml in RPMI medium for 30mins. After 30mins of incubation, all cells were washed twice with PBS to remove the BLM prior to being treated with aspirin and ibuprofen, each in nano form and the standard bulk form and

incubated for a further 30mins, except one set treated with bleomycin alone which processed the normal Comet without repair.

The purpose was to measure the influence of these compounds on the repair of DNA using the alkaline comet assay, and compared with two sets of samples; one post-bleomycin treatment and not repaired, and the other sample was incubated with complete RPMI-1640 medium and repaired without any NSAIDs in a $CO_2$ incubator for 30mins (37°C) to allow cells to repair damage induced by bleomycin. Different types of statistical analysis were used (see section 2.2.11.2 below).

### 2.2.11.2 Statistical analysis

Two slides were scored from each sample. At least 100 cells were scored randomly. The analysis was performed using a microscope equipped with a CCD camera and computer system using Comet Kinetic Imaging Software© 6.0 (Liverpool/Andor Technology, Belfast, UK). Each experiment was repeated 15 times independently for samples from healthy individuals and prostate cancer patients. Mean data and standard errors were generated.

Data obtained as Olive tail moment and % Tail DNA were tested for normality using the Kolmogorov-Smirnov test. The non-parametric Mann-Whitney U test was used to compare treated samples with the negative control values to obtain the P values. Data from Olive tail moment and % Tail DNA values were used to perform SPSS statistics (Version 20). P values of <0.05 were considered statistically significant. Significant differences *P<0.05; **p<0.01 ***p< 0.001 compared to (control) untreated lymphocytes. Significant differences ($^{+++}$ p = <

0.001, $^{++}$ p = < 0.01, $^{+}$ p = < 0.05) compared to treated with BLM. Significant differences ($^{\Delta\Delta\Delta}$ p = < 0.001, $^{\Delta\Delta}$ p = < 0.01, $^{\Delta}$ p = < 0.05) compared to self-repair.

### 2.2.12 Detection of reactive oxygen species (ROS)

1.5 x $10^5$ isolated lymphocytes were seeded in phenol red-free RPMI medium into 25$cm^2$ flasks. After 24hrs, cells were re-suspended in complete media without phenol red to a concentration of 1 x $10^6$ cells/ml and treated with both forms of aspirin. Another set, aspirin and TBHP, were simultaneously added to lymphocytes. Lymphocytes not treated with TBHP were considered as normal control and the second well was treated with 50µmTBHP and used as a positive control. One blank well with no cells, but with the compound, was incubated. Next, 1hr prior to completion of the treatment, cells were stained by adding the diluted 2ml of phenol red-free RPMI containing 25µM of 2',7' – dichlorofluorescein diacetate (DCFDA) (DCFDA, Abcam) at a concentration of 1 x $10^6$ cells/ml and incubated for 45mins in the dark at 37ºC. Cells were transferred to a dark bottom 96-well microplate with 100,000 stained cells/well and were measured on a fluorescence plate reader at Ex/Em=485/535nm. Carboxy-H2DCFDA is a chemically reduced, acetylated form of fluorescein used as an indicator for ROS in cells. Carboxy-H2DCFDA is readily converted to a green-fluorescent form when the acetate and ester groups are removed by intracellular oxidation (i.e. by the activity of ROS). The ROS change was determined as a percentage of control and treated wells after background subtraction.

#### 2.2.12.1 Statistical data analysis

The significance of results were assessed through a comparison of means using two-tailed student t-test. Results were expressed as the mean ± standard deviation. P values were calculated to determine statistical significance of the results.

# Chapter 3

# The genotoxicity of aspirin and ibuprofen bulk and nano particles on peripheral lymphocyte

## 3.1 Introduction

Epidemiological studies support the idea that prolonged inflammation, (chronic inflammation), contributes to the pathogenesis of various forms of human cancer (Crusz and Balkwill, 2015). Approximately 20% of cancers in adult humans reportedly result from chronic inflammatory conditions caused by infectious agents, chronic non-infectious inflammatory diseases, and other environmental factors. Furthermore, research also suggests that chronic inflammation plays a role in the aetiology of prostate cancer. In particular, recent research has focused on the following: (i) potential stimuli for prostatic inflammation; (ii) prostate cancer immunobiology; (iii) inflammatory pathways and cytokines in prostate cancer risk and development; (iv) proliferative inflammatory atrophy (PIA) as a risk factor for prostate cancer development; and (v) the role of nutritional or other anti-inflammatory compounds in reducing prostate cancer risk (Sfanos and De Marzo, 2012).

Some studies have linked chronic prostatitis with prostate cancer (Sfanos et al., 2014). Additional evidence linking inflammation and cancer comes from clinical studies of nonsteroidal anti-inflammatory drugs (NSAIDS) that found that long-term users of NSAIDS, including aspirin, have a reduced risk of developing prostate cancer (Jacobs et al., 2007).Furthermore, blocking either inflammatory mediators or signalling pathways that regulate inflammation decreases tumour frequency and delays tumour growth, while heightened levels of proinflammatory mediators or the adoptive transfer of inflammatory cells increases tumour development (Mantovani et al., 2008).

Chronic inflammation is believed to promote onset and progression through both immune and nonimmune mechanisms. The immune mechanism involves the

perturbation of myelopoiesis and hemopoiesis, which initiates a deficiency in antigen presenting (Ag-presenting) dendritic cells (DC) and dysfunctional cell-mediated antitumour immunity (Gabrilovich, 2004).

The non-immune mechanisms include the production of reactive oxygen species (ROS) which cause DNA damage, an initiating event leading to cancer (Eiró and Vizoso, 2012) .the production of pro-angiogenic factors for instance, vascular endothelial growth factor (VEGF), which stimulates tumour neovascularisation (Ellis and Hicklin, 2008) and the production of matrix metalloproteases, which are essential for promoting metastasis and invasion (Yang et al., 2008). DNA damage in somatic cells can potentially result in the development of cancer (Gopalan et al., 2011). Additionally, several factors can influence susceptibility to cancer, such as exposure to genotoxins, genome sensitivity and possibly the functionality of DNA repair mechanisms (Collins, 2004).

In humans, the Comet assay is used to explore genetic damage with the goal of assessing exposure to genotoxic agents from occupational hazard, drug treatments, and environmental pollution (Faust et al., 2004). It has also been used for DNA repair studies in radiation and chemical biology, for environmental bio-monitoring, and in genetic toxicology and human epidemiology (Faust et al., 2004). In the last decade, this assay has been used to examine the genotoxicity of nanoparticles (NPs) and has proven suitable for such measurements (Karlsson et al., 2015; Magdolenova et al., 2014).

In addition, the micronucleus assay (MN) is an essential test in genotoxicity for studying DNA damage at the chromosomal level, as chromosomal mutation is a crucial event in carcinogenesis (Fenech, 2007).

It has been proven that inflammatory processes can influence cancer development by inducing mutations, which cause further increases in genomic lymphocyte damage (Ben-Baruch, 2006). As all cells share the same DNA, lymphocytes were used in previous studies as surrogate cells to examine the degree of DNA damage (Anderson et al., 2014).

Additionally, for the last decade, lymphocytes have been used widely as cytogenetic biomarkers to survey genotoxic risks in work environments (Garaj-Vrhovac and Oreščanin, 2009).

Nano-medicine can potentially improve drug efficacy. Therefore, this study examines the effect of aspirin and ibuprofen in bulk and nanotised states on lymphocytes from both healthy volunteers and prostate cancer patients using the Comet assay and micronucleus assay to evaluate whether the increase in the activity of aspirin and ibuprofen by producing the nano sized of aspirin and ibuprofen could lead to an increase genetic damage or could confer a geno-protective effect.

## 3.2 Materials and methods

All chemicals used in the Comet assay and micronucleus assay are listed in Table 2.1.

The methods for the Comet assay are as described in chapter 2 section 2.2.7

For the micronucleus assay methods, please refer to chapter 2 section 2.2.8

## 3.3 Results

### 3.3.1 Particle size and stability

#### 3.3.1.1 Characterisation of NPs, bulk powder and their stability

Dynamic light scattering (DLS) is a standard technique used to measure the size-distribution profile of small particles in suspension (Hiroi and Shibayama, 2017). Accordingly, the particle sizes of aspirin and ibuprofen were measured using the DLS technique of the Zetasizer Nano ZS (Malvern Instruments, UK). After the sample was illuminated with a laser, fluctuations of the scattered light were analysed and the size of the particles was measured. DLS measurements were taken before and after cell treatment and at the monthly intervals for the nanotised version in order to avoid particle aggregation for both aspirin and ibuprofen nanoforms.

The zeta potential (ZP) was also measured in order to ensure the stability of the suspensions. ZP is a measure of the magnitude of the electrostatic potential between particles, with a higher ZP indicating a greater stability and greater ability to resist aggregation, while a low ZP indicates a tendency to flocculate. The initial mean particle size in aspirin nano-suspension (5%) was 289 ± 3nm with a polydispersity index of 0.3 ± 0.03 and a zeta potential of -6.1 mV, indicating that nanotised aspirin is relatively unstable and could potentially aggregate; therefore,

new suspensions were prepared monthly. Moreover, the mean particle size distribution (Z-average) in the ibuprofen nano-suspension (4%) was 323 ± 6.4 nm with a polydispersity index of 0.2 ± 0.01 and a ZP of -2.1 mV, indicating that the ibuprofen was more stable. The particle size distributions of the cells before and after treatment with both the aspirin and ibuprofen nano-suspensions were 299 ± 6.3 nm, and 340 ± 1.2 nm with polydispersity indexes of 0.3 ± 0.05 and 0.3 ± 0.001, respectively.

The mean particle sizes of aspirin and ibuprofen bulk powders was determined using laser diffraction (Sympatec Helos, UK) (see Table 3.1).

| **Suspension name** | **Average particle size (µm)** | **Volume Mean Diameter(µm)** |
|---|---|---|
| **Ibuprofen** | 52.80 ± 4.37 | 20.50 |
| **Aspirin** | 78.30 ± 0.23 | 44.57 |

Table 3.1 Average particle size and volume mean diameter of the aspirin and ibuprofen bulk powder (n=3). This table was provided by Dr Mohammed Isreb, School of Pharmacy, University of Bradford.

### 3.3.1.2 Transmission electron microscopy

Transmission electron microscopy (TEM) is a microscopy technique that creates detailed images of tiny objects by transmitting a beam of electrons through an ultra-thin specimen, interacting with the specimen as it passes through. The TEM image of aspirin nanoparticles demonstrates their nearly spherical shape and their size of 289 ± 3 nm. The images showed that aspirin crystals are larger than those of ibuprofen (see Figures 3.1 and 3.2).

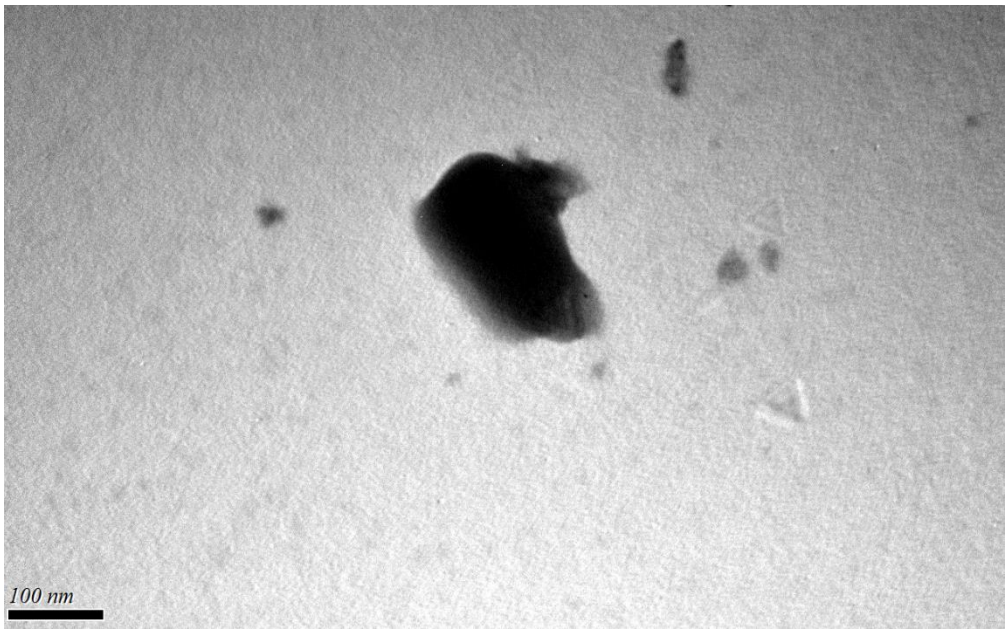



Figure 3.1. TEM image of ibuprofen nanoparticles

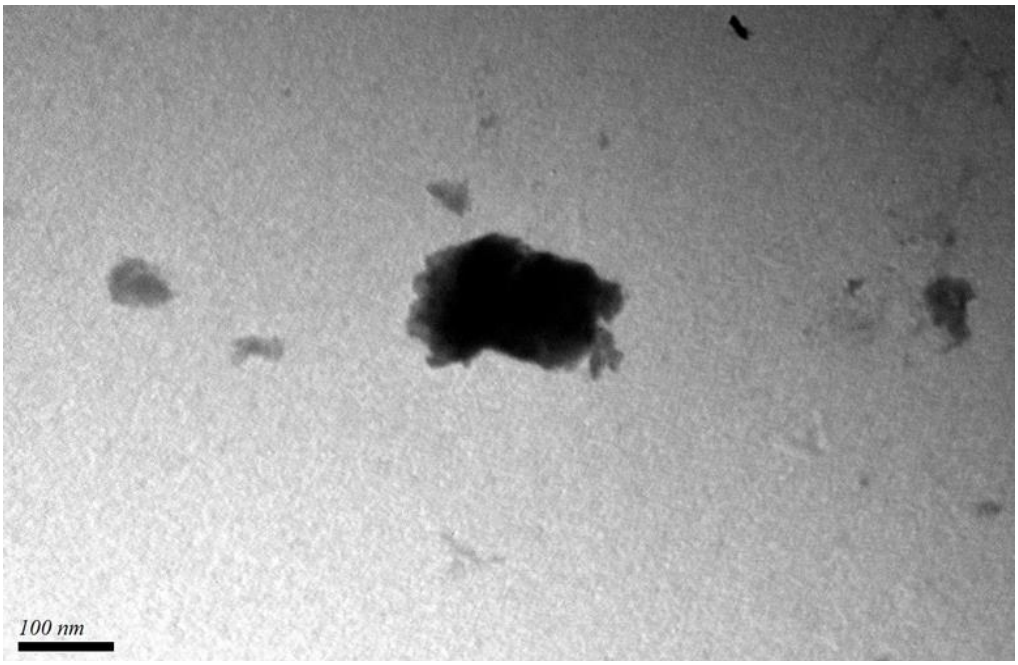



Figure 3.2 TEM image of aspirin nanoparticles, indicating the nearly spherical shape**.**

### 3.3.2 The effect of aspirin and ibuprofen, bulk and nano particles on lymphocytes DNA from healthy volunteers.

The Comet assay (Alkaline version) was used to investigate the effect of bulk and nanoform aspirin and ibuprofen on lymphocytes from healthy individuals. Bulk and nanotised preparations were examined to determine if a decrease in particle size induced DNA damage. Results are showed as Olive Tail Moment (OTM) and the percentage of DNA in the tail.

As shown in figures 3.3 and 3.4, our data from the OTM indicate that exposure to aspirin bulk (ASP B), aspirin nanosuspension (ASP N), ibuprofen bulk (IBU B), and ibuprofen nanosuspension (IBU N) caused a reduction in DNA damage, as compared to an untreated control, in lymphocytes from healthy donors, only the IBU N, ASP B and ASPN were significant ($p< 0.01$, 0.01 and 0.001, respectively). In both drugs tested, the reduction of the DNA damage of the nanosuspension was greater than that of bulk form. The ASP N induced a significant decrease in DNA damage compared to ASP B. Also, IBU N showed a significant reduction in DNA damage compared to IBU B (Figure 3.3 and 3.4; Table 3.2).

The maximum reduction in Olive Tail Moment (OTM) and percentage of Tail DNA was around 1.3-fold and 6.1-fold respectively in healthy cells treated with ASP N compared to untreated control. Indicating that the reduction in the particle size for aspirin and ibuprofen had a significant effect. ASP N was the most effective compound compared to ASP B form, and both forms of ibuprofen. The reduction of the DNA damage seen in aspirin from the bulk to the nano form was less than of that of ibuprofen; a similar trend was seen in the percentage of DNA in the tail (Figure 3.4 and Table 3.2).

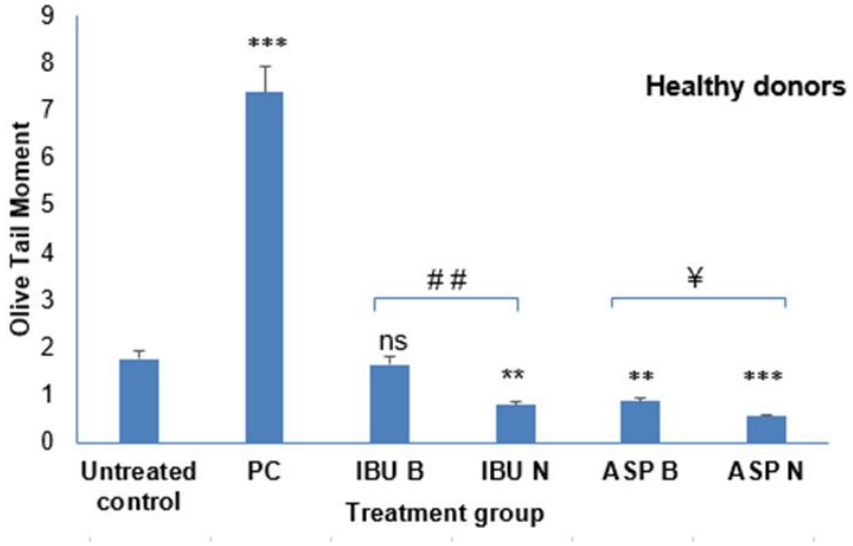



**Figure 3.3. Effects of bulk and nanotised aspirin and ibuprofen preparations on lymphocyte DNA from healthy individuals using OTM**. Figure shows mean of Olive Tail Moments for the effect of five different groups of treatment, ibuprofen bulk (IBU B), ibuprofen nano (IBU N), aspirin bulk (ASP B), aspirin nano (ASP N), the positive control (PC) $H_2O_2$ and an untreated lymphocyte. The DNA damage was measured immediately after treatment by alkaline comet assay in human lymphocyte cells obtained from 20 healthy individuals. Data are expressed as mean±SEM. Statistical significance was calculated and illustrated after comparing 1) untreated control score values with values obtained with all treatment groups. 2) IBU N suspension was compared to bulk suspension. 3) ASP N suspension was compared to bulk suspension. *$P$< 0.05 compared with the negative control group. $^{\#}P$< 0.05 compared IBU B with IBU N and $^{¥}P$< compared ASP N with ASP B. ns, non-significant, $^{*}$P<0.05; $^{**}$P<0.01 $^{***}$P< 0.001.

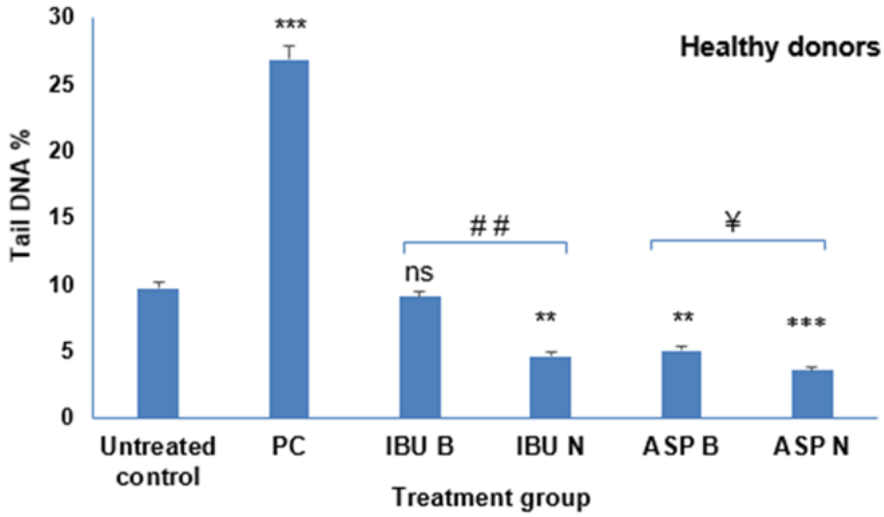



**Figure 3.4. Effects of aspirin and ibuprofen bulk and nanotised preparations on lymphocytes DNA from healthy individuals using percentage of Tail DNA**. Figure shows mean of percentage of Tail DNA for five different groups of treatment ibuprofen bulk (IBU B), ibuprofen (IBU N), aspirin bulk (ASP B), aspirin nano (ASP N), the positive control (PC) $H_2O_2$, and an untreated lymphocyte group in human lymphocyte cells obtained from 20 healthy individuals. All treatment groups were compared with the negative control group, ASP N suspension was compared to bulk suspension and IBU N suspension was compared to bulk suspension. All data have been expressed as means and standard error (SE); asterisks (*) represents significant differences between five different groups of treatment $^{*}$P<0.05; $^{**}$p<0.01 $^{***}$p<0.001. *P < 0.05, untreated control versus all the treatment group. $^{\#}P$< 0.05, IBU B versus IBU N. $^{¥}P$< 0.05, ASP B versus ASP N.

Table 3.2 Olive Tail Moment and Percentage of Tail DNA in Healthy Donors.

| **Treatment** | **Olive Tail Moment Mean ± SEM** | ***P* value** | **percentage of Tail DNA Mean ± SEM** | ***P* value** |
|---|---|---|---|---|
| Untreated | 1.8 ± 0.16 | - | 9.6 ± 0.4 | |
| $H_2O_2$ | 7.3 ± 0.5 | ***p<0.001 | 26.8 ± 0.9 | ***p<0.001 |
| Ibuprofen Bulk (IBU B) | 1.6 ± 0.15 | ns | 9.0 ± 0.43 | ns |
| Ibuprofen Nano (IBU N) | 0.7 ± 0.09 | **p<0.01 ##p<0.01 | 4.5 ± 0.28 | **p<0.01 ##p<0.01 |
| Aspirin Bulk (ASP B) | 0.8 ± 0.09 | **p<0.01 | 5.0 ± 0.27 | **p<0.01 |
| Aspirin Nano (ASP N) | 0.5 ± 0.03 | ***p<0.001 ¥p<0.05 | 3.5 ± 0.20 | ***p<0.001 ¥p<0.05 |

¥= P < 0.05 ASP N suspension compared to ASP B.
##= P <0.01 IBU N suspension compared to IBU B.

### 3.3.3 Treatment of lymphocytes of prostate cancer patients with aspirin and ibuprofen bulk and nanoforms

The primary aim of this study was to evaluate the effect of aspirin and ibuprofen bulk and nanotised preparations to examine their anti-cancer efficacy further to determine their potential mechanisms of action. Results from the OTM from the prostate cancer patients (Figure 3.5) show a significant reduction in DNA damage with ASP B ($p$ <0.01), ASP N ($p$<0.001) and IBU N (p<0.01) when compared to the untreated controls (table 3.3). In aspirin and ibuprofen, the nanotised version exhibited a significant decreased damage to the DNA when compared to bulk counterpart 1.3-fold and 2-fold, respectively (Figure 3.5). Also, Aspirin was the most effective agent, with both bulk and nano formulation exceeding that of the Ibuprofen. A similar trend was seen in the percentage of DNA in the tail (Figure3.6) with a significant decrease observed in ASP B (p <0.01), ASP N (p<0.001) and IBU N (p<0.01).

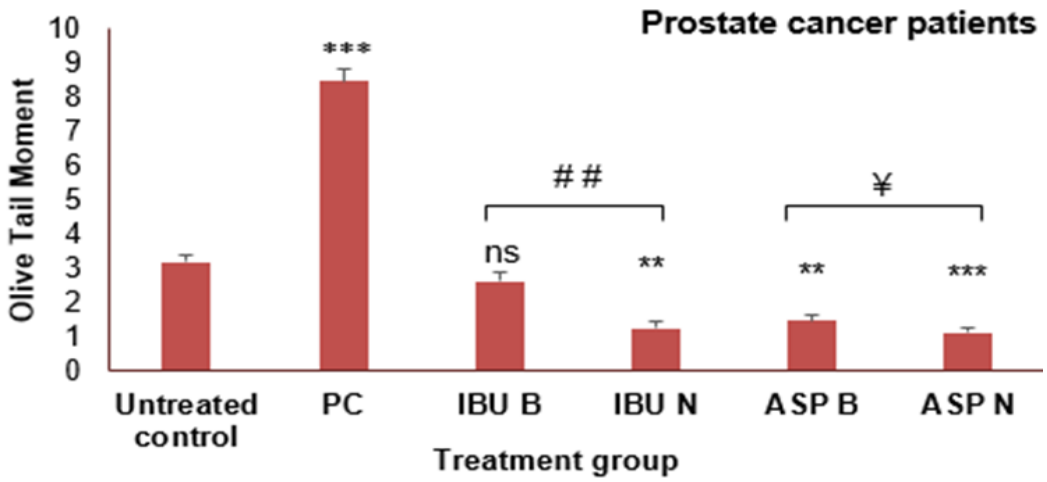



**Figure 3.5. The effect of bulk and nanotised-NSAIDs on lymphocytes DNA from prostate cancer patients using OTM**. The figure shows five different groups of treatment ibuprofen bulk (IBU B), and ibuprofen nanoform (IBU N), aspirin bulk (ASP B), aspirin nanoform (ASP N) and the positive control (PC) $H_2O_2$, and an untreated lymphocyte group in human lymphocyte cells obtained from 20 prostate cancer patients. All treatments were compared with the negative control. The mean values for the Olive Tail Moments obtained from twenty experiments involving 100 cells each. The positive control (50 µM $H_2O_2$) had a maximum mean 8.4 value for Olive Tail Moments. All data have been expressed as means and standard errors (SEs); asterisks (*) represent significant differences between untreated control and the five different groups of treatment $^{*}p < 0.05$; $^{**}p < 0.01$ $^{***}p < 0.001$. $^{\#}$ P< 0.05 represent significant between IBU B and IBU N and $^{¥}$P< represent significant differences between ASP N and ASP B.

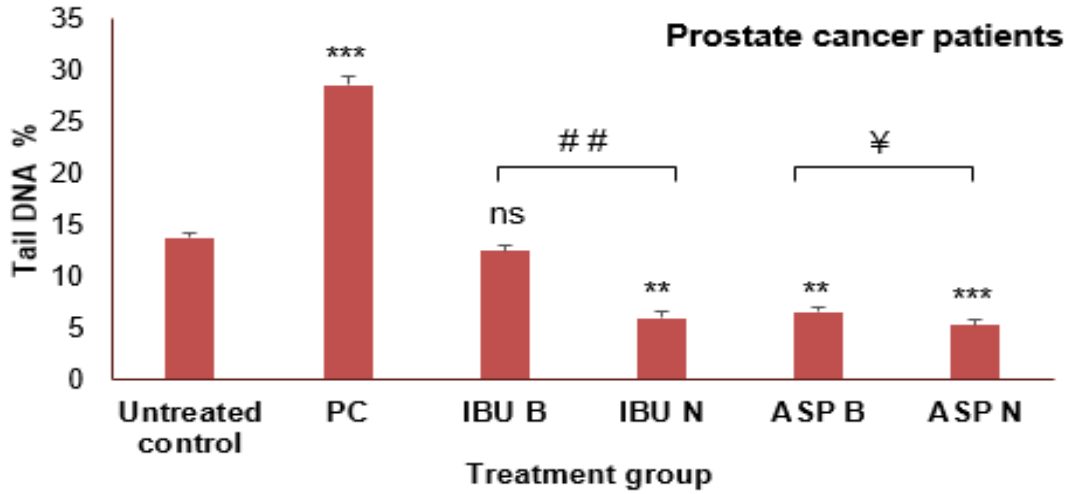



**Figure 3.6. The effect of bulk and nanotised NSAIDs on lymphocytes DNA from prostate cancer patients using the percentage of Tail DNA.** The figure shows the mean of percentage of Tail DNA for five different groups of treatment ibuprofen bulk (IBU B), and ibuprofen nanoform (IBU N), aspirin bulk (ASP B), aspirin nanoform (ASP N) and the positive control (PC) $H_2O_2$ and an untreated lymphocyte group in human lymphocyte cells obtained from 20 prostate cancer patients. All treatments were compared with the negative control group. The positive control (50 µM $H_2O_2$) had a maximum mean value of 28.5 for percentage of Tail DNA. All data are expressed as means ± standard error (SE); asterisks (*) represents significant differences between five different groups of treatment compared to untreated control $^{*}p < 0.05$; $^{**}p < 0.01$ $^{***}p < 0.001$. (#) represent significant between IBU B and IBU N and (¥) represent significant differences between ASP N and ASP B.

Table 3.3 OTM and percentage Tail DNA in prostate cancer patients

| Treatment | Olive Tail Moment Mean ± SEM | *P* value | Percentage of Tail DNA Mean ± SEM | *P* value |
|---|---|---|---|---|
| Untreated | 3 ± 0.2 | - | 13.7 ± 0.4 | - |
| $H_2O_2$ | 8.4 ± 0.36 | ***P <0.001 | 28.5 ± 0.76 | ***P <0.001 |
| Ibuprofen Bulk (IBU B) | 2.6 ± 0.24 | ns | 12.47 ± 0.52 | ns |
| Ibuprofen Nano (IBU N) | 1.2 ± 0.19 | **P<0.01 ##P <0.01 | 6.0 ± 0.66 | **P<0.01 ##P <0.01 |
| Aspirin Bulk (ASP B) | 1.4 ± 0.17 | **P<0.01 | 6.5 ± 0.54 | **P<0.01 |
| Aspirin Nano (ASP N) | 1.1 ± 0.14 | ***P<0.001 ¥P <0.05 | 5.3 ± 0.45 | ***P<0.001 ¥P <0.05 |

¥P <0.05 ASP N suspension compared to bulk suspension

##P <0.01 IBU N suspension compared to bulk suspension

### 3.3.4 Comparing the effect of aspirin and ibuprofen (NPs and bulk) on lymphocytes DNA from prostate cancer patients and healthy individuals.

A difference between healthy donors and prostate cancer patients was observed when the level of DNA damaged was assessed in untreated lymphocytes: the cancer patients showed a significantly higher level of DNA damage on lymphocytes than healthy donors (***p<0.001). Also, DNA damage decreased in lymphocytes from healthy individuals and prostate cancer patients after treatment with ASPB, ASPN, IBU B and IBU N. However, this reduction was significant with ASPB, ASPN and IBU N. Furthermore, healthy individual saw a significant reduction of DNA damage with ASP B (**$p < 0.01$), ASPN (***$p < 0.001$), IBU B (***$p < 0.001$), and IBU N (**p < 0.01) when compared to prostate cancer patients treated with ASP B,ASP N, IBU B AND IBU N respectively (Figure 3.7). This result indicates that lymphocytes from prostate cancer patients exhibited more DNA damage than healthy lymphocytes.

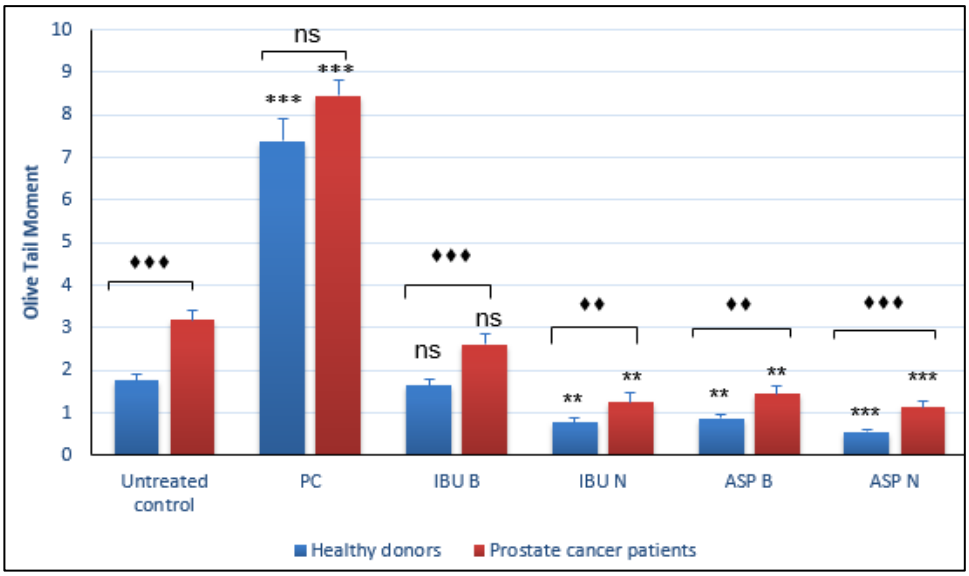



**Figure 3.7. Comparing aspirin and ibuprofen (NPs and bulk) effects on lymphocyte DNA from prostate cancer patients and healthy individuals**. The effect of both aspirin and ibuprofen, bulk and nanotised forms, on DNA in lymphocyte cells obtained from healthy individuals compared to prostate cancer patients as measured by the Comet assay parameter using OTM. Cells were treated with both aspirin and ibuprofen in both forms at concentrations of 500 µg/ml, with a negative control of untreated lymphocytes and a positive control of 50 µM of $H_2O_2$ for 30 minutes. All data is expressed as means ± standard error (SE); asterisks (*) represent significant differences between five different groups of treatment $^{*}p<0.05$; $^{**}p<0.01$ $^{***}p< 0.001$ compared to untreated control**.** (♦) represent significant differences between the DNA levels of healthy individual compared to prostate cancer patients.

### 3.3.5 Analysis of Confounding Factors

The effect of the variables studied (age, ethnicity, drinking habits, smoking habits) on comet values were evaluated in both the healthy donor group and prostate cancer group. In general, we found no relationship among the confounding factors in any of the treatment groups (see Table 3.4).

#### 3.3.5.1 Age

Tables 3.5 and 3.6 show that the two oldest patient age groups (65-75 and > 70) expressed similar results, exhibiting higher basal DNA damage (♦$p < 0.001$) than patients in the 55-65 age group, who nevertheless featured significant basal damage (♦$p < 0.05$) in comparison to the two untreated age controls.

ASP N treated lymphocytes showed decreased DNA damage in terms of OTM according to patient age: prostate cancer patients between 55-65 expressed

a significant ($p < 0.001$) decrease in DNA damage, followed by patients between 65-75 ($p < 0.05$), and patients < 75 ($p < 0.05$). ASP B treated lymphocytes showed a decrease in DNA damage in all age groups. However, this decrease was only significant in the 55-65 patient age group, which showed a significant ($p < 0.01$) reduction in DNA damage compared to untreated controls. Treatment with IBU N showed a significant reduction in DNA damage ($p < 0.05$) in patients in the 65-75 and < 75 age groups. However, treatment with IBU B showed no significant differences in all age groups. In addition, there was agreement between OTM and % of tail DNA.

#### 3.3.5.2 Ethnicity

Tables 3.5 and 3.6 demonstrate no statistically significant differences between healthy Asian patients after treatment with IBU B and ASP B when compared to untreated controls. However, ASP N and IBU N showed a significant reduction in DNA damage. There was also a significant reduction in DNA damage in Caucasian patients after lymphocyte treatment with IBU B, IBU N, ASP B, and ASP N. In addition, there were no statistically significant differences between the lymphocytes of Asian and Caucasian cancer patients. Moreover, the Caucasian control participants not differed from the Asian control participants after treatment with ASP B and ASP N (see Table 3.4).

#### 3.3.5.3 Drinking Habits

There was a statistically significant reduction of prostate cancer DNA damage in lymphocytes treated with IBU N, ASP B, and ASP N compared to untreated lymphocytes. We found no relationship between drinking habits and comet parameters nor increased or decreased DNA damage in any of the groups (see Table 3.4).

### 3.3.5.4 Smoking Habits

Tables 3.5 and 3.6 demonstrate that patients who smoked showed the most significant DNA damage for both comet parameters; the non-smoking controls had the lowest damage when compared with both smoking and nonsmoking patients. Smoking patients had the highest DNA baseline damage (♠$p < 0.001$), followed by nonsmoking patients (♦$p < 0.05$). Further, ASP N showed a significant reduction (***$p < 0.001$) in DNA damage in lymphocytes in smoking patients, followed by ASP B (**$p < 0.01$) and IBU N (*$p < 0.05$), when compared to untreated lymphocytes in smoking patients.

**Table 3.4: Confounding Factors**

| **Confounding Factors** | **p value** |
|---|---|
| Age | $p < 0.85$ |
| Ethnicity | $p < 0.47$ |
| Drinking habits | $p < 0.701$ |
| Smoking habits | $P < 0.33$ |

**Table 3.4. The effect of confounding factors on DNA damage.** The table shows the relationship between the age, ethnicity, drinking habits, smoking habits on DNA damage evaluated in both the healthy donors and prostate cancer patients.

**3.5**

| Group type and confounder | | NC | PC | IBU B | IBU N | ASP B | ASP N |
|---|---|---|---|---|---|---|---|
| **Age** | Controls < 50 | 0.75 ± 0.3 | 6.9 ± 2 *** | 1.6 ± 0.5 ns | 0.6 ± 0.2 ns | 1.4 ± 0.5 ns | 0.6± 0.19ns |
| | Controls 55-65 | 1.5 ± 0.5 ns | 7.1 ± 2.2*** | 1.4 ± 0.8 ns | 0.7 ± 0.2 ns | 0.89 ± 0.3 ns | 0.50 ± 0.1 ns |
| | Patients 55-65 | 2.7 ± 0.7 ♦ | 8.3 ± 0.9*** | 2.1 ± 0.7 ns | 0.95 ± 0.5 ** | 1.18 ± 0.7 ** | 0.8 ± 0.3*** |
| | Patients 65-75 | 3.1 ± 1.3♠ | 9.0 ± 2.7*** | 2.7 ± 1.4 ns | 1.1 ± 0.4* | 1.2 ± 0.5 ns | 0.9 ± 0.2 * |
| | Patients >75 | 3.5 ± 0.8♠ | 7.9 ± 1.5 *** | 2.7 ± 1.4 ns | 1.3 ± 1.2 * | 1.5 ± 0.8 ns | 1.1 ± 0.7 * |
| **Smoking History** | Non-smoking Controls | 1.0 ± 0.6 | 7.3 ± 3.3 *** | 1.9 ± 1.1 ns | 0.8 ± 0.66 ns | 1. 9 ± 0.8 ns | 0.5 ± 0.18 ns |
| | Smoking Controls | 1.2 ± 1.4 ns | 9.7 ± 5.8 *** | 2.8 ± 3.2 *** | 1.3 ± 2 ns | 2.3 ± 2.6 ns | 1.2 ± 0.6 ns |
| | Non-smoking Patients | 2.4 ± 0.8 ♦ | 8.4 ± 2.2 *** | 2.6 ± 0.6 ns | 1.0 ± 0.56 ns | 1.8 ± 0.5 ns | 1.1 ± 0.2 ns |
| | Smoking patients | 3.0 ± 1.0 ♠ | 8.3 ± 1.7 *** | 2.5 ± 1.3 ns | 1.1 ± 0.9 * | 1.1 ± 0.5 ** | 0.9 ± 0.5 *** |
| **Ethnicity** | Caucasian controls | 1.2 ± 0.1¤ | 5 ± 1.5 *** | 1 ± 0.3 ns | 0.6 ± 0.07 ns | 0.6 ± 0.08 ns | 0.4 ± 0.05 ns |
| | Caucasian patients | 3 ± 0.9¤ | 8.5 ± 1.6*** | 2.6 ± 1* | 1± 0.8 ** | 1.5 ± 0.7 ** | 1± 0.6*** |
| | Asian controls | 1.7 ± 0.8 | 7.5 ± 2.4*** | 1.7 ± 0.7 ns | 0.7 ± 0.4* | 0.9 ± 0.4ns | 0.6 ± 0.18 ** |
| | Asian patients | 3.7 ± 1 | 7.7 ± 1.4*** | 3 ± 1.5 ns | 2 ± 1.3 ns | 2 ± 0.6 ns | 1.9 ± 0.8 ns |
| **Drinking habit** | Drinking Controls | 1.9 ± 0.88 | 7.6 ± 2.9*** | 1.7 ± 0.8 ns ¤ | 1 ± 0.5 ns ¤ | 0.8 ± 0.5 ns ¤ | 0.6 ± 0.2 ns ¤ |
| | Non-drinking Controls | 1.65 ± 0.7 | 7 ± 2 *** | 1.6 ± 0.6 ns | 0.6 ± 0.1 * | 0.87 ± 0.4 ns | 0.5 ± 0.1 * |
| | Drinking Patients | 3 ± 0.97♦ | 8 ± 1.6*** | 2.6 ± 1ns ¤ | 1.3 ± 0.8 ** ¤ | 1.4 ± 0.7 ** ¤ | 1 ± 0.6*** ¤ |
| | Non-drinking Patients | 3 ± 1♠ | 8.8 ± 1.7*** | 2.6 ± 1 ns | 1 ± 0.5** | 1 ± 0.5 ** | 0.8 ± 0.3*** |

**3.6**

| Group type and confounder | | NC | PC | IBU B | IBU N | ASP B | ASP N |
|---|---|---|---|---|---|---|---|
| Age | Controls < 50 | 8.7 ± 1.9 | 27 ± 4.6 *** | 8.3± 1.6 ns | 4.5 ± 1.4 ns | 5.9 ± 2.2 ns | 3.5 ± 1 ns |
| | Controls 55-65 | 10 ± 2.6 | 28 ± 3.8 *** | 8.8 ± 1.5 ns | 4.8 ± 1.4 ns | 4.7 ± 1.4 ns | 3.7 ± 0.6 ns |
| | Patients 55-65 | 13 ±2.6 ♦ | 29 ± 3*** | 11 ± 2 ns | 5.6 ± 3.7 ** | 6.9 ± 2.9** | 4 ± 1.6*** |
| | Patients 65-75 | 13.7 ± 1 ♠ | 28 ± 3*** | 12.7 ± 2 ns | 6 ± 3.5 * | 6.5 ± 2.9 ns | 5.5 ± 2.5 * |
| | Patients >75 | 13.9 ± 2 ♠ | 28 ± 4*** | 12.9 ± 2.8 ns | 5.5 ±1.4 * | 5 ±1 ns | 4.7 ± 1* |
| Smoking History | Non-smoking Controls | 8.0 ± 1.9 | 26 ± 5*** | 9 ± 1.5 ns | 4.7 ± 1.3 ns | 5.5 ± 2 ns | 3.8 ±1.1 ns |
| | Smoking Controls | 9.6 ± 2.5♠ | 28.8 ± 3.6 *** | 10 ± 1.6 ns | 4.5 ± 1.7 ** | 6.1 ± 2.3 ** | 3.2 ± 0.6 *** |
| | Non-smoking Patients | 12 ± 1.1 ♦ | 28.7 ± 3.7 *** | 12 ± 1.5 ns | 5.3 ± 3 ns | 7.8 ± 4 ns | 5 ± 1.8 ns |
| | Smoking patients | 14 ± 2.3 ♠ | 29 ± 1.9*** | 12 ± 2.6 ns | 5.5 ± 2.5** | 5.6 ± 1.5*** | 4.8 ± 1.8 *** |
| Ethnicity | Caucasian controls | 8.6 ± 1.2 | 23.9 ± 5*** | 9 ± 1.3 ns | 4 ± 1.7 ns | 4.9 ± 1.3ns | 3 ± 0.3 ns |
| | Caucasian patients | 13.7 ± 2 | 28.5 ± 3*** | 12.5 ± 2* | 6 ± 2 ** | 6.5 ± 2 ** | 5 ± 2*** |
| | Asian controls | 9.7 ± 1.9 | 26.8 ± 4.5 *** | 9 ± 2 ns | 4.6 ± 1* | 5 ± 1.3 ns | 3.5 ± 0.9 ** |
| | Asian patients | 14.6 ± 2 | 23.5 ± 2 *** | 13.6 ± 3 ns | 9 ± 3 ns | 8.5 ± 1.7 ns | 7.6 ± 2 ns |
| Drinking habit | Drinking Controls | 8.9 ± 1.6 ▫ | 25 ± 4.7*** | 7.9 ± 2 ns | 4.5 ± 1 ns | 4.9 ± 0.8 ns | 3.5 ± 1 ns |
| | Non-drinking Controls | 10 ± 2 ▫ | 28 ± 3.6*** | 9.9 ± 1.4 ns | 4.6 ± 1.4 * | 5 ± 1.5 ns | 3.6 ± 0.8* |
| | Drinking Patients | 13.8 ± 2 ♦ | 28.5± 3.4 *** | 12.5 ± 2.4 ns | 6 ± 3 ** | 6.5 ± 2.5** | 5± 2 *** |
| | Non-drinking Patients | 14 ± 1.6 ♠ | 29.5 ± 2.7 *** | 12.5 ± 2 ns | 5.3 ± 1.9** | 5.7 ± 1** | 4.6 ± 1*** |

**Tables 3.5 and 3.6. The effect of the age, ethnicity, drinking habits, smoking habits on DNA damage.** Both tables show Mean ± SD of Olive tail moment (OTM) and Mean ± SD of % of tail DNA in the lymphocytes of healthy controls and prostate cancer patients were divided by the confounding factors. In each group, the mean OTM of treated lymphocytes was compared with the mean OTM of untreated control lymphocytes (Nc); ANOVA (*$p < 0.05$, **$p < 0.01$, ***$p < 001$) and ns (not significant) were not found to be significant (▫$p < 0.05$, ♦$p < 0.01$, ♠$p < 0.001$) when patients and confounding factors were compared with corresponding control samples. The positive control (Pc) 50µM $H_2O_2$ was included.

### 3.3.6 The effect of human lymphocyte treatment with aspirin and ibuprofen bulk and nano formulation in the cytokinesis block micronucleus assay (CBMN).

Lymphocytes from prostate cancer patients and healthy individuals were treated with 200µg/ml of IBU B, IBU N and 500 µg/ml of ASP B, ASP N then tested for the induction of micronuclei (MNi) using cytokinesis block micronucleus assay. The results are presented in Table 3.7. The dose concentration tests showed that 200 µg/ml of ibuprofen and 500 µg/ml of aspirin were the optimum doses to use in the experiments, without inducing cell cytotoxicity.

#### 3.3.6.1 The frequency of binucleated cells (BiNC), multinucleated cells (MultiNC) and nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) after treatment with NSAIDs.

For both the healthy individuals and prostate cancer patients group, see Table 3.7. The aspirin and ibuprofen bulk and nanotised formulation increased the number of BiNC compared to untreated controls. However, this increase was not significant. The number of MultiNC decreased 1, 5.3, 5.5, and 4-fold after treatment with ASP B, ASP N. IBU B and IBU N respectively in prostate cancer lymphocytes when compared to untreated healthy cells (Table 3.7). The Nuclear Division Index (NDI) ranged from 1.74 to 1.92 in healthy individuals and from 1.70 to 2.00 in prostate cancer patients, which were within the normal range limits of 1.3 to 2.2 (Fenech, 2007). The frequency of NPBs and NBUDs were also within the normal range (Table 3.7).

### 3.3.6.2 The micronuclei (MNi) frequency after treatment with NSAIDs.

In the present experiment, the number of MNi in 1,000 binucleated cells was determined. The frequency of induced MN was used as an indicator of DNA damage (Fenech, 2007).

**In the healthy individual group**: The number of MNi in the aspirin nanoform treated cells were at the same level as in untreated cells from healthy individuals. The treatment of the lymphocytes from the healthy individuals with IBU N, ASP N, or ASP B caused a significant reduction in the MNi formation ($P \leq 0.001$) compared to untreated lymphocytes from prostate cancer patients (Table 3.7 and Figure 3.8). There was an increase in the frequency of MNi of BiNC cells treated with bulk ibuprofen when compared to the negative control. The data demonstrated that the IBU B treatment caused a significant increase in MNi formation.

**In the prostate cancer group**: The data showed a significant 9-fold increase in the MNi frequency in the untreated prostate cancer lymphocytes compared to untreated healthy lymphocytes. The number of MNi after the treatments with ASP N and ASP B decreased by 4.5-fold and 2-fold, respectively, compared to the untreated lymphocytes from the prostate cancer patients ($p < 0.05$ and $p \leq 0.001$, see Figure 3.8). In aspirin, the nanotised version saw a 4.5-fold greater decrease in the MNi frequency when compared to the bulk counterpart. The prostate cancer lymphocytes treated with nanotised ibuprofen showed MN numbers that were almost the same levels as those of the negative control group.

The data obtained from the healthy donors treated with bulk ibuprofen had a similar pattern to that of the prostate cancer patients (Table 3.7). The present data demonstrated that treatment with aspirin in nanotised and bulk form did not

induce chromosome aberrations, as indicated by the decrease in MNi formation within the bionucleated cells. The data also, indicated that a decrease in the particle size of aspirin does not cause any genotoxicity.

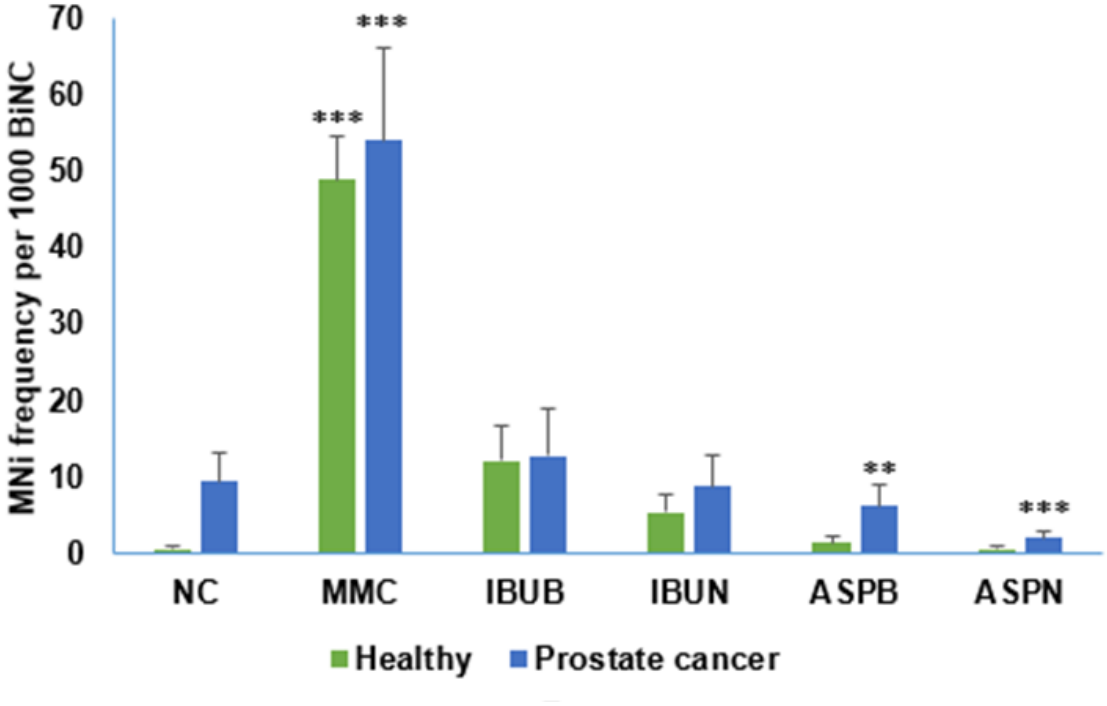



**Figure 3.8. The average of BiNC scored per 1000 cells per culture from five independent experiments**; n = 1000. All data have been expressed as means ± standard deviations (SD); and compared to untreated lymphocytes (NC) to calculate the significance differences. One asterisk (*), two asterisks (**) and three asterisks (***) represent significant differences between NC untreated lymphocytes, PC (MMC), IBU B, IBU N and ASP B and ASP N.

| Subject | Treatment Group | NDI | % BiNC | % Multi | per 1000 BiNC cells | | | % MoNC |
|---|---|---|---|---|---|---|---|---|
| | | | | | BiMNi | BiNPB | Bi Buds | MNi |
| **Healthy Individuals** | Untreated Lymphocytes | 1.92 | 59 | 16.5 | 1 | 0 | 0 | 3 |
| | 0.4 μM MMC | 1.80 | 63 | 11 | 59 | 0 | 1 | 22 |
| | ASP B | 1.85 | 66 | 10 | 1 | 0 | 0 | 1 |
| | ASP N | 1.75 | 68 | 4.9 | 0.5 | 0 | 0 | 0 |
| | IBU B | 1.74 | 67 | 3 | 12 | 0 | 1 | 6 |
| | IBU N | 1.85 | 73 | 6 | 5 | 0 | 0 | 2 |
| **Prostate cancer patients** | Untreated | 1.90 | 65 | 15 | 9 | 0 | 0 | 7 |
| | 0.4 μM MMC | 1.70 | 64 | 5.4 | 54 | 0 | 0 | 27 |
| | ASP B | 2.00 | 69.5 | 15.5 | 6 | 0 | 0 | 3 |
| | ASP N | 1.80 | 70 | 3 | 2 | 0 | 0 | 2 |
| | IBU B | 1.77 | 71 | 3 | 13 | 0 | 1 | 9 |
| | IBU N | 1.83 | 76 | 4 | 8 | 0 | 0 | 4 |

**Table 3.7. Average of different types of cells in the cytokinesis block micronucleus assay, including BiNC, MoNC, and MultiNC.** The average of (NDI) and binucleated, mononucleated, bionucleated with MNi (BiMNi) nucleoplasm bridges (NPBs) and nuclear buds (NBUDs) in lymphocytes following treatment with both bulk and nanotised forms of aspirin and ibuprofen. Untreated lymphocytes of prostate cancer patients showed an increased number of MNi compared to samples treated with ASP B, ASP N and IBU N. In general, Table 3.7 shows that the number of MNi in both prostate cancer patients and healthy individuals declined more in the nanotised form of aspirin and ibuprofen compared to the bulk form, indicating that nanotised compounds decrease DNA damage.

## 3.4 Discussion

All NSAIDs act through the inhibition of cyclooxygenase (COX) enzyme activity. COX is known to play a significant role in the biosynthesis of prostaglandins (e.g. $PGE_2$), which can exacerbate the progression of cancer (Antonio et al., 2015) and, consequently, tumour development (Day and Graham, 2013; Rao and Knaus, 2008). Several studies have shown that NSAIDs, especially aspirin and ibuprofen, can influence the hallmarks of cancer, such as cell proliferation, evasion of apoptosis, and cell cycle regulation (Burn et al., 2012; Park et al., 2014).

However, none of the previous studies on these NSAIDs has tested in vitro the effect of both aspirin and ibuprofen, in bulk or nanoform, on the DNA of lymphocytes from prostate cancer patients. Genetic defects in DNA repair may contribute to higher levels of DNA damage in lymphocytes and target tissue in cancer patients (Hanahan and Weinberg, 2000). Because obtaining normal primary prostate epithelial cells from healthy individuals is not possible, lymphocytes are the focus of this study.

The alkaline Comet assay used in this study is known for its simplicity, sensitivity, time efficiency, and cost effectiveness for assessing DNA integrity in cells (Gopalan et al., 2011).

The Comet assay data showed that the basal level of DNA damage in prostate cancer patients is significantly higher than in healthy donors in both OTM and percentage of Tail DNA. This result agrees with a previous study that found that in many cases, cancer basal level of DNA damage in a cancer patient is higher than in a healthy individual (Anderson et al., 2014).

Our results also showed, in both the healthy group and the prostate cancer group, that exposing lymphocytes to ASP B, ASP N, IBU B, and IBU N caused a decrease in DNA damage compared to the negative controls in OTM and percentage of Tail DNA (Figures 3.3, 3.4, 3.5 and 3.6) and this reduction was significant in the prostate cancer group. However, this decrease was not significant in healthy lymphocytes. The ASP N induced a significant decrease in DNA damage compared to the ASP B. in fact the ASP N was the most effective compound in inducing a significant decrease in DNA damage compared to the bulk aspirin (Figure 3.3, 3.4).

The results demonstrate the ability of NSAID compounds to reduce DNA damage, especially with the increased surface reactivity of nanoparticles that stems from their large surface area to volume ratio. This finding was in line to some extent with a previous study that revealed a geno-protective effect of, aspirin when co-administrated with ~~a~~ mitomycin C a known genotoxic agent (Niikawa et al., 2008).

One study showed that administration of aspirin to mice before treatment with a carcinogen Ochratoxin dramatically reduced the number of DNA adducts in the urinary bladder and kidney (Obrecht-Pflumio et al., 1996). A similar effect was observed when aspirin suppressed the genotoxicity of mitomycin C (MMC) in a somatic mutation and recombination test (SMART) in *Drosophila melanogaster* (Niikawa et al., 2006).

Moreover, an ibuprofen and thiamine combination possesses a significant chemoprotective effect in diethylnitrosamine-induced hepatocellular carcinoma in Wistar rats (Afzal et al., 2017). Using the Comet assay, one study found that ibuprofen shows no genotoxic effect on whole human blood (Manosij et al., 2010).

This reduction in nanotoxicity is potentially important, as it suggests that nanotising particles leads to a probable increase in the reactivity of drugs.

These results, however, were in contrast with a study showing that ibuprofen induces dose dependent genotoxicity in the bone marrow of mice (Tripathi et al., 2012).

Another study found that aspirin shows weak genotoxicity in the bone marrow of mice when evaluating sister chromatid exchanges and chromosomal aberrations at the highest dose tested (Giri et al., 1996).

It is known that some personal characteristics and habits, such as, age, sex, or drinking and smoking habits, may modulate the effect of anti-inflammatory drugs on DNA damage or repair. In the present study, we found no correlation between multiple extrinsic variables and the comet results, suggesting that the observed DNA alterations were mainly due to the effect of the aspirin bulk, ibuprofen bulk, and aspirin and ibuprofen nano forms. This finding was in line to some extent with a previous study by Najafzadeh et al. (2016) found that there were no significant differences between the results in relation to confounding factors such as gender, smoking, drinking habit, ethnicity and age.

The cytokinesis-block micronucleus is an important test to measure the ability of genotoxic agents to induce clastogenic and aneugenic effects on cell divisions and cell cycles (Fenech, 2002).

In this study, lymphocytes were cultured, treated in vitro with aspirin and ibuprofen in bulk and nanotised forms and evaluated for a possible expression of MNi; nucleoplasm in both bridges and nuclear buds resulting from chromosome breakage and/or a disturbance of chromosome segregation are indicators of genomic instability.

NDI is a measure of general cytotoxicity and thus a marker of cell proliferation, as a considerable degree of chromosomal damage causes a reduction of NDI. In both healthy volunteers and prostate cancer patients, the NDI percentages were found to be within the normal expected range of 1.3 to 2.2, as seen in (Table 3.7), indicating a successful division of cells.

Also, the NDI for prostate cancer patients was lower than for healthy donors, indicating genomic instability. Further, the MNi frequency was a significantly higher in untreated prostate cancer patients than in the healthy donors, and this is in agreement with a previous study that found that MNi in lymphocytes from lung cancer patients were significantly higher than in those of healthy individuals (El-Zein et al., 2006; Lou et al., 2007).

Furthermore, as compared to the untreated control, treatment with bulk and nanotised aspirin decreased MNi frequency in lymphocytes from prostate cancer patients. This result is in agreement with studies that have reported that the genotoxicity of analgesic compounds assessed by an in vitro micronucleus assay, indicating that aspirin failed to induce micronuclei in the normal rat-kidney cell line NRK-49F (Antunes et al., 2007; Dunn et al., 1987; Oldham et al., 1986). Additionally, in short-term cytogenetic tests in normal human lymphocyte cultures treated with aspirin, no significant increase in chromosomal aberrations was observed (Antunes et al., 2007). However, nanotised aspirin was capable of decreasing the formation of MNi more than the bulk form; this might be related to the surfaces of nanoparticles, which should be taken into account due to possible electrostatic interactions between the surface of nanoparticles and cellular proteins. This conjecture may have important implications for the relationship between the potential effects of nanomaterials and their surface modifications

(Xia et al., 2006). MNi formation increased after treatment with bulk and nanotised ibuprofen, and this result contradicted the Comet assay result. This might be due to differences in the exposure time of both assays as the Comet assay had an incubation time of 30 min while the MN assay had a more extended incubation time of 72 hours.

# Chapter 4

# The protective effect of NSAIDs against oxidative damage and quantification of DNA repair capacity in peripheral lymphocytes from healthy individuals and prostate cancer patients.

## 4.1 Introduction

Cancers are associated with pronounced genomic instability when compared with normal tissues in the same patient. However, several studies have shown that genomic instability is raised not only in tumour tissue but also in non-cancer cells of cancer patients, and especially in peripheral lymphocytes (PBLs) (Orlow et al., 2008; Santos et al., 2010). These previous studies suggested that the grade of genome damage found in PBLs indicates an individual's tendency to develop cancer.

The DNA repair machinery is essential for maintaining and preserving genome integrity. Consequently, impairment of the ability to efficiently repair damaged DNA is strongly associated with high cancer risk in humans (Moller et al., 2000; Zheng et al., 2003). This opinion was first supported by the discovery that rare autosomal recessive genetic disorder diseases, such as Fanconi anaemia, Bloom's syndrome, ataxia-telangiectasia (A-T) and Xeroderma pigmentosum (XP) (Berwick and Vineis, 2000; Hemminki et al., 2000), are commonly associated with genomic instability, a lack of DNA repair and an increased risk of developing cancer (Zheng et al., 2003).

The capacity to repair DNA damaged by endogenous or exogenous genotoxic agents, such as oxidation, also differs widely among individuals (Berwick and Vineis, 2000; Hemminki et al., 2000). For these reasons, the epidemiology of DNA repair capacity and its influence on cancer incidence in humans has become an essential area in cancer research.

A considerable number of epidemiological studies have compared the variations in DNA repair capacity between cancer patients and healthy controls to evaluate the role of repair in the progression of human cancer (Berwick and Vineis, 2000;

Hemminki et al., 2000; Mohrenweiser and Jones, 1998). For example, previous research by Dybdahl et al. (1999) showed that a high capacity to repair damaged DNA is crucial for protection of psoriasis patients against chemically induced basal cell carcinoma. Similarly, Zheng et al. (2003) reported that a low capacity to repair damaged DNA has a strong association with lung cancer risk, while Ramos et al. (2004) concluded that a lack of DNA repair capacity was a predisposing factor in breast carcinoma.

DNA repair processes are classified into numerous pathways: homologous recombination repair (HRR), non-homologous end-joining (NHEJ), base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MR) (Bernstein et al., 2002; Mohrenweiser and Jones, 1998). At present, five different types of techniques have been used to evaluate DNA repair capacity.

The first includes tests based on induction of DNA damage with chemicals or physical agents, such as the G2-radiation assay, mutagen sensitivity test and the Comet and micronuclei assays. The second involves the use of indirect methods for measuring DNA repair, such as un-programmed DNA synthesis (UDS). The third type incorporates tests based on more direct measures of repair kinetics, such as the host cell reactivation assay (HCR). The fourth type assesses the correlation between genetic variation and DNA repair and the fifth integrates more than one type of assay (Hemminki et al., 2000).

Of these various techniques, the Comet assay stands out as a highly sensitive, simple assay for detecting all kinds of DNA damage, including single-strand and double-strand breaks and alkali-labile lesions (Fairbairn et al., 1995; Vandghanooni and Eskandani, 2011). The combination of the Comet assay and a mutagen challenge test can be used for indirect determination of an individual's

DNA repair capacity (Duthie et al., 2002; Marcon et al., 2003). Bleomycin (BLM) is a radiomimetic agent that can produce single-strand and double-strand breaks by composing a complex with ferrous ions and molecular oxygen, resulting in generation of oxygen radicals at the site of DNA intercalation (Pastwa et al., 2001).

One potent cause of DNA damage is exposure to reactive oxygen species (ROS). These are generally tiny molecules, but are highly reactive. They are generated as natural products of normal cellular metabolism and include free radicals, such as superoxide anion ($O2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$), as well as non-radical molecules like hydrogen peroxide ($H_2O_2$) and singlet oxygen ($^1O_2$) (Birben et al., 2012). At low levels, ROS have positive effects on cellular functions; however, exposure to environmental stress can lead to overproduction of ROS. High ROS levels in living cells are associated with significant adverse modifications and damage to macromolecules, including proteins, lipids and DNA, and a cell under these conditions is considered to be in a state of oxidative stress (Valko et al., 2006; Wang et al., 1996).

Oxidative stress is caused by an imbalance between ROS production and scavenging. It occurs in a broad spectrum of human diseases, such as chronic inflammation, age-related disease and cancer (Durackova, 2010).

An increase in ROS levels has been linked to the promotion of tumour cell metastasis through increases in vascular permeability, while treatment with ROS scavengers can significantly reduce the metastatic potential of tumours in mice (Brown and Bicknell, 2001).

ROS can also modulate cellular signalling to activate cellular processes such as proliferation, differentiation, inflammation responses and cell survival through multiple ROS-sensitive signalling pathways, thereby maintaining the cancerous phenotype of the cell (Marinho et al., 2014).

A shift in the balance between oxidants and antioxidants toward a more oxidative state is seen in prostate cancer. Some studies have highlighted the changes in oxidant and antioxidant status in prostatic tissues and cell lines and have indicated that an imbalance between these two antagonists can initiate prostate carcinogenesis (Khandrika et al., 2009; Ripple et al., 1997).

The mechanism underlying this imbalance has not been elucidated, but the relationship with cancer suggests that the use of anti-inflammatory drugs, which can relieve oxidative stress, may be useful in the prevention or treatment of some types of cancer.

The role played by anti-inflammatory drugs in the prevention of the cancer is still poorly understood, but potentially involves their effects on cellular oxidative status. One way to study the oxidant: antioxidant balance in cells is with tert-butyl hydroperoxide (T-BHP), a well-known inducer of ROS that is commonly used to evaluate the cellular injury due to oxidative stress (Kucera et al., 2014).

## 4.2 Aims

The objectives of this study were to test the hypothesis that anti-inflammatory drugs, specifically aspirin and ibuprofen, can facilitate DNA repair. The approach taken was to use bleomycin (BLM) challenge tests, combined with Comet assays, to measure DNA repair capacity in lymphocytes from prostate cancer patients and healthy individuals following treatment with both bulk and nanoformulated forms of aspirin and ibuprofen. We used T-BHP to induce oxidative damage in these lymphocytes to investigate the potential protective effects of the bulk and nanoformulated aspirin to obtain a better understanding of the antioxidant potency of aspirin in the blood system.

## 4.3 Materials and methods

All chemicals used in this experiments are listed in chapter 2 (Table 2.1)

The methods for bleomycin challenge assay is described in chapter 2 section 2.2.11.

For human lymphocyte isolation methods, please refer to methods in chapter 2 section 2.2.9.1.

The method for Detection of reactive oxygen species (ROS) is described in chapter 2 section 2.2.12.

## 4.4 Results

### 4.4.1 The effect of bulk and nano forms of aspirin and ibuprofen on DNA repair capacity in bleomycin-pretreated peripheral blood lymphocytes from healthy individuals and prostate cancer patients

BLM is commonly used to study DNA damage and repair (Schmezer et al., 2001; Wu et al., 2007). It works by binding to the DNA strands and causing single and double-stranded DNA breaks. This BLM experiment involved different treatment groups. The first group consisted of peripheral lymphocytes (PBLs) from healthy donors and prostate cancer patients; these were pretreated with BLM alone for 30 mins prior to Comet assays for measurement of the levels of DNA damage. The second group consisted of similar lymphocytes pretreated with BLM for 30 mins; however, in this case, the BLM was removed and replaced with either aspirin or ibuprofen (bulk and nanoformulated) and incubated for an additional 30 mins prior to DNA repair capacity measurements with the Comet assay.

And the third group sample pretreated with BLM for 30 mins, in this case, BLM was replaced with complete RPMI-1640 medium and repaired without any

NSAIDs (self repair) in a $CO_2$ incubator for 30mins (37°C) to allow cells to repair damage induced by bleomycin. PBLs without treatment (untreated), PBLs treated with BLM only and PBLs repaired without any NSAIDs (self repair) were used as controls. The results are shown in Figures 4.1 (A&B) and 4.2 (C&D).

In general, the Comet assay results for Olive tail moments (OTMs) and % Tail DNA revealed similar trends for PBLs from prostate cancer patients and from healthy individuals in response to NSAID treatment. However, we noted a much higher baseline DNA damage (nearly 2.5-fold higher) in PBLs from prostate cancer patients than in PBLs from healthy (non-cancer) donors.

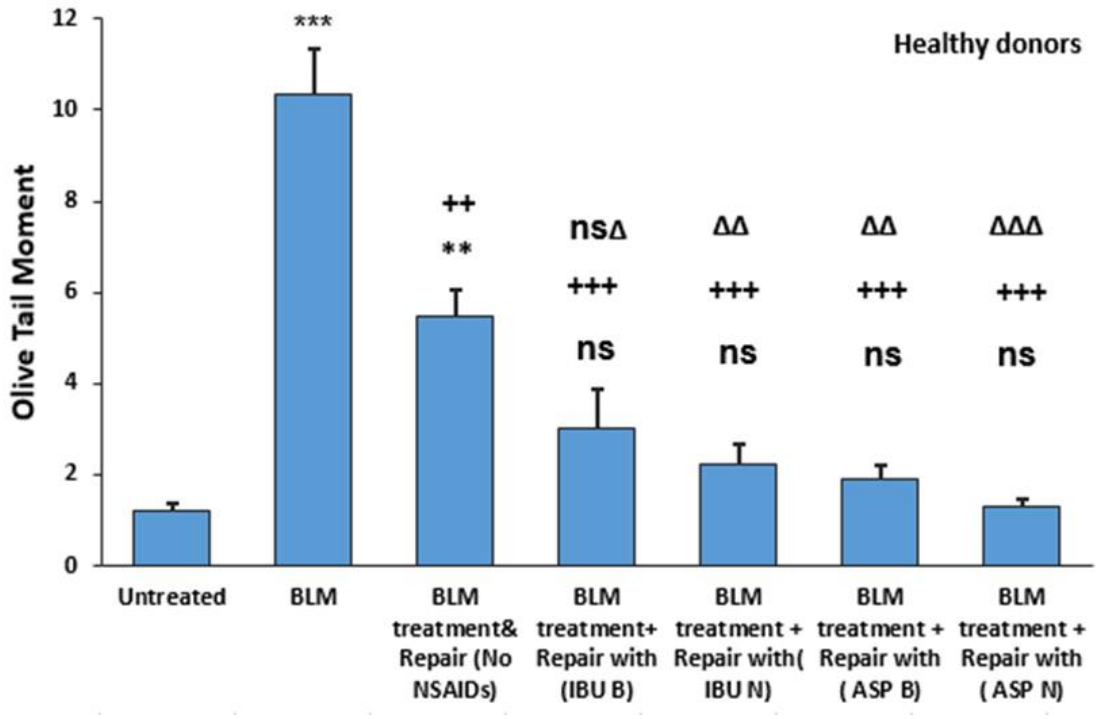
The OTM data also indicated a significantly higher (***$p < 0.01$) induced DNA damage in PBLs from healthy individuals and prostate cancer patients after a 30 min treatment with BLM alone, when compared to the untreated control PBLs (Figure 4.1 A, 4.2 C). However, we found differences between the treatment groups when we removed the BLM to provide a 30 min repair period and evaluated the subsequent DNA damage repair capacity.

The PBLs from healthy donors showed a significant reduction in the level of DNA damage within 30 mins of removal of the BLM and treated with ASP B, ASPN, IBU B and IBU N ($^{+++}p < 0.01$) when compared to PBLs treated with BLM with no repair period (Figure 4.1A). However, no significant (∆**ns**) DNA damage reduction was detected in these lymphocytes from prostate cancer patients following treatment with bulk ibuprofen (IBU B) when compared to PBLs treated with BLM with no repair period (Figure 4.2 C and D).

Comparison of the NSAID-treated versus the treated PBLs with bleomycin and allowing DNA repair in the absence of NSAIDs (self-repair) (Figures 4.1-4.2 A&B,

C&D), showed a significant decline in bleomycin effects in the PBLs from healthy donors and prostate cancer treated with both forms of aspirin (ASP B) ($^{\Delta\Delta}p<0.01$) (ASP N) ($^{\Delta\Delta\Delta}p<0.001$) and with nanoformulated ibuprofen (IBU N) ($^{\Delta\Delta}p<0.01$). The nanoformulated aspirin showed the best reduction ($^{\Delta\Delta\Delta}p<0.001$) compared with self-repair. The bulk form of ibuprofen also reduced the DNA damage, but the reduction was not statistically significant when compared with self-repair in PBLs from healthy individuals and prostate cancer patients. The reduction in DNA damage in PBLs from healthy donors was nearly 1-fold, 1.5-fold and 1.8-fold lower in response to nanoformulated and bulk aspirin and nanoformulated ibuprofen, respectively, when compared to the corresponding control value. The reduction was nearly double that noted in PBLs from prostate cancer patients.

**A**



**B**

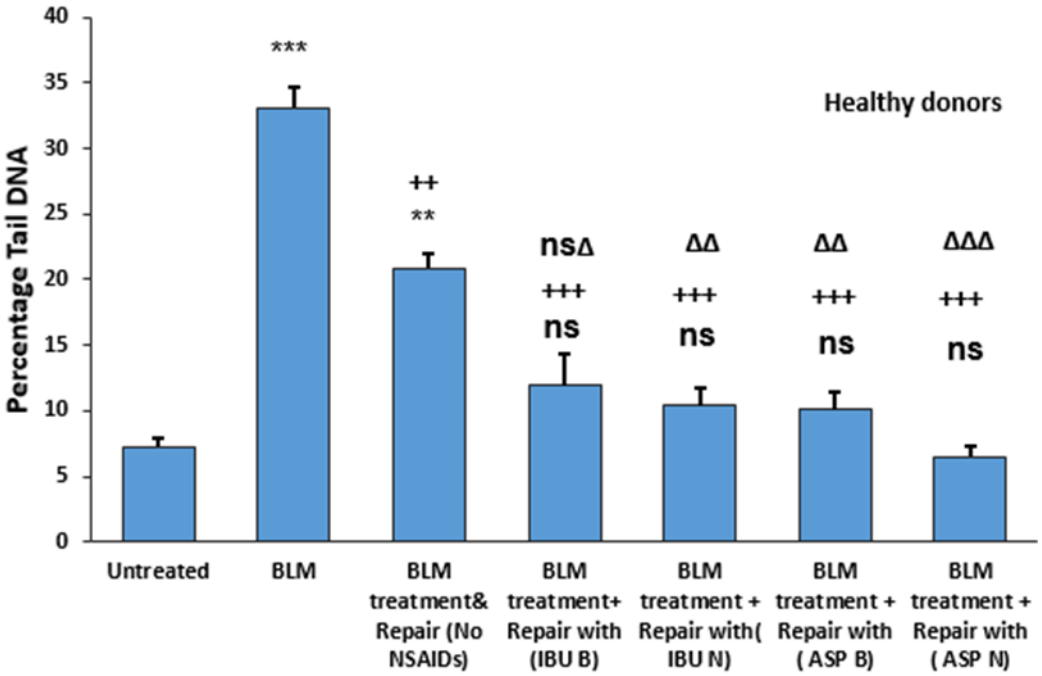



**Figure 4.1(A & B): The effect of aspirin and ibuprofen bulk and nanoformulation after a pre-treatment challenge with bleomycin.** A and B show the effect of bulk and nanoformulated aspirin (ASP B, ASP N, respectively) and ibuprofen (IBU B, IBU N, respectively) after a challenge with bleomycin in peripheral blood lymphocytes from prostate cancer patients using Olive tail moment (A) and % Tail DNA (B) in the comet assay. The data were analysed by one-way ANOVA followed by a Tukey test to determine significant differences. (*) represents significant differences between all the treatment groups compared to control untreated lymphocytes. 2) ($^{\Delta}$) represent significant differences between all treatment groups compared to self-repair. 3) ($^{+}$) represent significant differences between all treatment groups compared to bleomycin. *P<0.05; **p<0.01 ***p< 0.001, ($^{+++}$ p = < 0.001, $^{++}$ p = < 0.01, $^{+}$ p = < 0.05), and ($^{\Delta\Delta\Delta}$ p = < 0.001, $^{\Delta\Delta}$ p = < 0.01, $^{\Delta}$ p = < 0.05) indicate significances. Errors bars represent SEM.

### 4.3.2. In *vitro* measurement of bleomycin-induced DNA damage by alkaline comet assays of lymphocytes from prostate cancer patients.

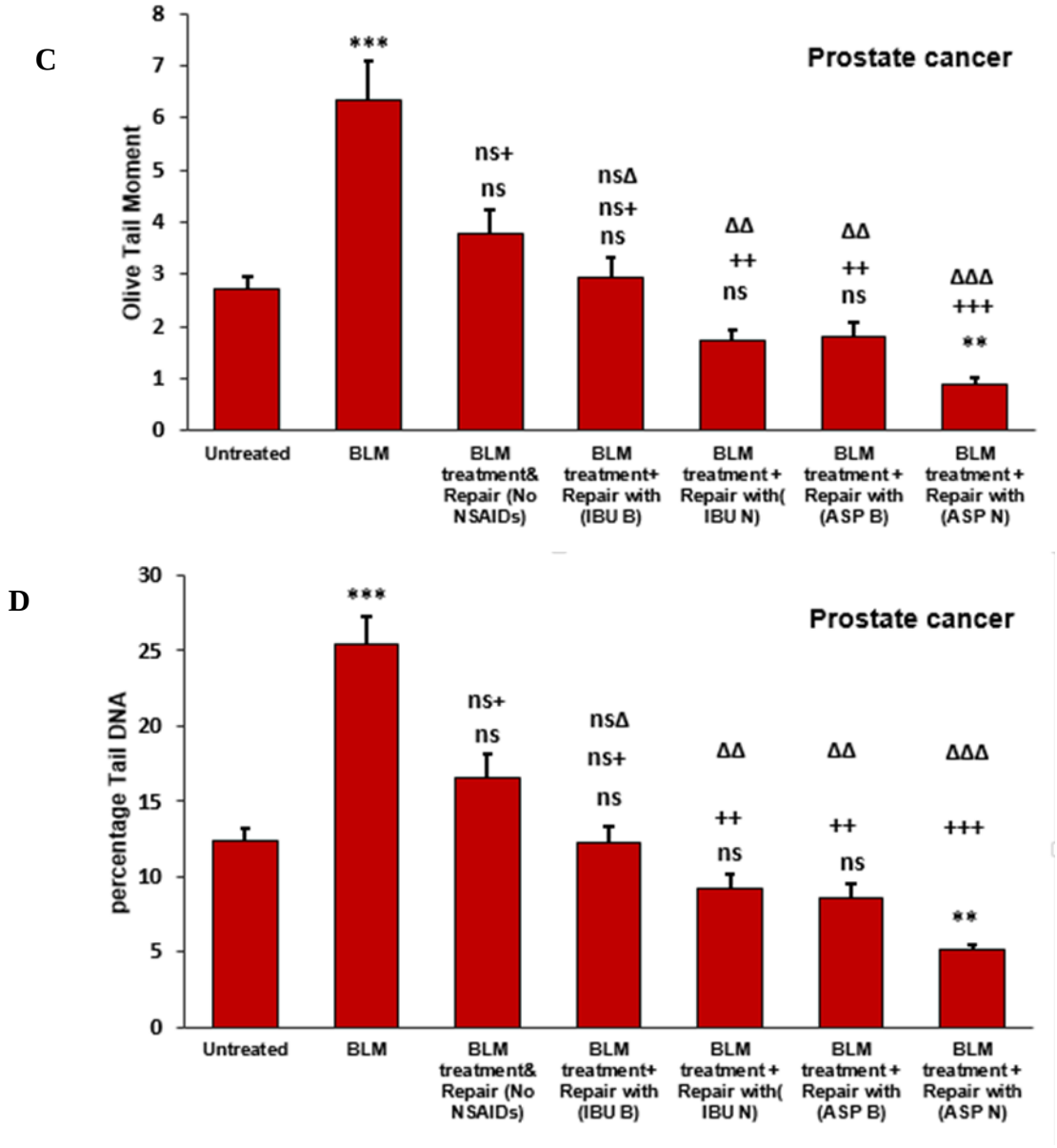



**Figure 4.2 (C&D) The effect of aspirin and ibuprofen after a challenge with bleomycin.** C & D show the effect of bulk and nanoformulated aspirin (ASP B, ASP N, respectively) and ibuprofen (IBU B, IBU N, respectively) after a challenge with bleomycin in peripheral blood lymphocytes from prostate cancer patients using Olive tail moment (C) and % Tail DNA (D) in the comet assay. The data were analysed by one-way ANOVA followed by a Tukey test to determine significant differences. . (*) represents significant differences between all the treatment groups compared to control untreated lymphocytes. 2) ($^{\Delta}$) represent significant differences between all treatment groups compared to self-repair. 3) ($^{+}$) represent significant differences between all treatment groups compared to bleomycin. Errors bars represent SEM.

### 4.4.2 DNA repair percentage (DRP) in lymphocytes from healthy individuals and prostate cancer patients measured with a bleomycin challenge assay

Figure 4.3 shows that lymphocytes from healthy individuals were more effective in DNA repair than were lymphocytes from prostate cancer patients. DNA repair percentage (DRP) values were used to compare the DNA repair capacities between different treatment groups. The repair capacity within the first 30 min after removal of BLM (self-repair) was lower in PBLs from prostate cancer patients (41%) than in PBLs from control subjects (47%).

Healthy PBLs treated with ASP N showed the highest DNA repair, with approximately 88% of the DNA damage repaired within 30 mins when compared to ASP N-treated PBLs from prostate cancer patients (85% DNA in a tail moment); however, these differences were not statistically significant. Most importantly, we observed a 70–87.5% recovery of BLM-induced DNA damage in 70% of the healthy individuals and in 41–85% of the prostate cancer patients (Figure 4.3). This result suggests that the aspirin enhanced repair capacity of lymphocytes.

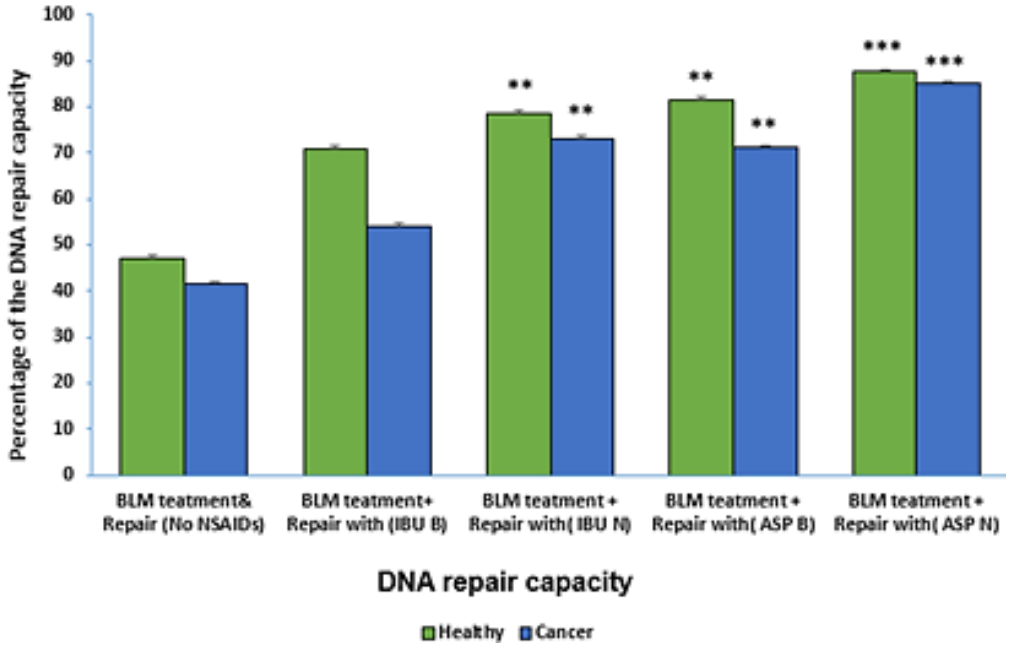



**Figure 4.3** The range of calculated DNA repair capacity measured as olive tail moment (OTM) among lymphocytes from healthy donors and prostate cancer patients after treatment with bleomycin, bleomycin + bulk ibuprofen (IBU B), bleomycin + nanoformulated ibuprofen (IBU N), bleomycin + bulk aspirin (ASP B) and bleomycin + nanoformulated aspirin (ASP N).
***p< 0.001 **p< 0.01 and *p< 0.05) indicate significances. Errors bars represent SEM.

### 4.4.3 Aspirin in nanoformulated and bulk form inhibits generation of reactive oxygen species (ROS)

The Cellular Reactive Oxygen Species Detection Assay utilises DCFDA, a fluorogenic dye that quantifies the activity of hydroxyl, peroxyl and other reactive oxygen species (ROS) within the cell. Upon diffusion into the cell, DCFDA is cleaved to DCF by cellular esterases into a non-fluorescent compound that can then be oxidised by ROS into 2', 7'–dichlorofluorescein (DCF). DCF is a highly fluorescent compound, and the fluorescence can be detected and measured. Several antioxidants can decrease intracellular ROS and inhibit the metabolism of DFC (Wolfe and Liu, 2007). For this reason, the inhibition of DCF can then be used to measure the intracellular ROS scavenging activity of antioxidants. T-BHP is widely used to evaluate the mechanisms of cellular modifications initiated by oxidative stress in cells and tissues (Fatemi et al., 2013).

We examined whether nanoformulated and bulk aspirin can affect intracellular ROS production. We used DCFDA staining to evaluate T-BHP-induced oxidative stress in PBLs from prostate cancer patients and healthy individuals. The PBLs were exposed to aspirin (bulk and nanoformulated) alone or in combination with 100µM T-BHP. We noted that T-BHP significantly increased the intracellular reactive oxygen species level compared to basal levels. All treated samples showed inhibition of ROS generation when compared to PBLs treated with T-BHP alone (Figure 4.4). Remarkably, treatment with aspirin alone significantly reduced ROS generation to values lower than the control values, and treatment with nanoformulated aspirin was the most effective at inhibiting ROS generation.

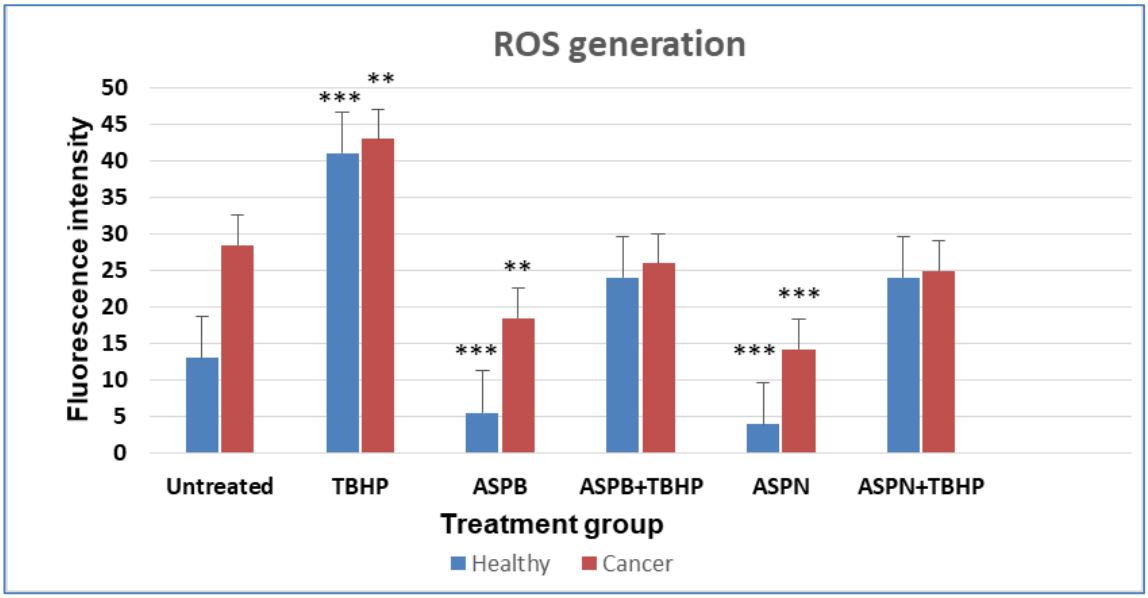



**Figure 4.4 Detection of reactive oxygen species (ROS) generation in lymphocytes after treatment with aspirin in bulk and nanoformulated forms**. The amount of ROS present in normal and experimental lymphocytes. Formation of ROS in normal and treated lymphocytes. Cont: untreated lymphocytes; T-BHP: lymphocytes treated with tertiary butyl hydroperoxide (T-BHP) (toxin control); ASPB & ASPN: lymphocytes treated with bulk aspirin (ASP B) and lymphocytes treated with nanoformulated aspirin (ASP N); ASP B + T-BHP: lymphocytes treated with aspirin before T-BHP intoxication; ASP N & T-BHP: lymphocytes treated with T-BHP and ASP N simultaneously. Each column indicates ±SD, n=3. Data were analysed by one-way ANOVA, with Student–Newman–Keuls post hoc tests. Differences between untreated control and treatment groups were attributed at *p<0.05; **p<0.01 and ***p< 0.001 indicate significant differences.

## 4.5 Discussion

An inability to maintain genomic integrity is a major risk factor in carcinogenesis. Increases in genomic instability, either spontaneous or mutagen-induced, have been regarded as susceptibility factors for malignant transformation (Zheng et al., 2003). Consequently, enhancing the DNA repair capacity is crucial for cell survival and maintenance of cell cycle control. Considerable inter-individual differences have been noted for the initial capacity for repair of DNA damage in various studies using in vitro lymphocyte assays.

Several lines of evidence have suggested that variation in the capacity of cells to repair DNA damage among individuals reflects individual genetic background variation. Various subpopulations of lymphocytes from the same individuals show the identical capacity to repair DNA damage, and the intra-individual difference in the repair capacity is significantly smaller than the difference among individuals (Mohrenweiser and Jones, 1998; Schmezer et al., 2001). Therefore, measuring DNA repair by peripheral lymphocytes can reveal an individual's overall DNA repair capacity.

Several studies have shown that a defect in DNA repair capacity is an independent risk factor for different kinds of cancer, including prostate cancer (Blasiak et al., 2004; Frenzilli et al., 2000; Hjertvik et al., 1998; Marcon et al., 2003; Moller et al., 2000; Tice et al., 2000; Zheng et al., 2003). For example, Popanda et al. (2003) found that deficiency in DNA repair is strongly associated with an increased risk of developing breast cancer, and Wu et al.(2003) showed that patients with lung cancer had a significantly lower DNA repair capacity than was observed in healthy controls.

Udumudi et al. (1998) reported that lower DNA repair capacity is a principal factor for cervical carcinoma risk.

Measurement of DNA strand breaks using Comet assays has been widely applied to assess genotoxicity, to monitor potentially carcinogenic exposures, or to evaluate DNA damage and repair in molecular epidemiology (Collins and Horvathova, 2001; Olive and Banath, 2006).

The comet assay, in particular, has been applied to evaluate the correlation of DNA repair capacity in peripheral lymphocytes with the risk of different kinds of cancers, including prostate cancer (Schabath et al., 2003; Schmezer et al., 2001). The results of our study indicated that lymphocytes from prostate cancer patients' show pronounced genomic instability that leads to an increased basal level of DNA damage and low DNA repair capacity (Figure 4.2 C, D and 4.3).

Baseline levels of DNA damage have been reported as significantly higher in breast cancer patients than in healthy donors (Santos et al., 2010). Kurzawa-Zegota et al. (2012) noticed greater baseline DNA damage in lymphocytes from colon cancer patients than from non-cancers donors, and this greater level of damage was similarly detected during in vitro treatment with genotoxic agents. Najafzadeh et al. (2012) showed that peripheral lymphocytes from patients with malignant melanoma and colorectal cancer, or their precancerous states, were far more sensitive to genetic mutagenesis than were lymphocytes from control participants. Schmezer et al. (2001) did not find any significant differences between the baseline levels of DNA damage in lymphocytes from lung cancer patients than from healthy donors, but they found an increased sensitivity to bleomycin and defects in DNA repair capacity in the lymphocytes from cancer patients. A similar lack of DNA repair has been detected in some studies on

lymphocytes from patients with lung, head and neck cancers (Altieri et al., 2008; Saha et al., 2008; Spitz et al., 2003; Walczak et al., 2012).

No consensus currently exists on the reasons for the genomic instability observed in lymphocytes from cancer patients. One possibility is that the genomic instability in lymphocytes is due to the generation of reactive oxygen species, as elevated levels were reported in the blood of various cancer patients (Diakos et al., 2014; Kryston et al., 2011).

A body of evidence now supports the occurrence of oxidative stress in breast, colon and lung cancer patients (Atukeren et al., 2010; Obtulowicz et al., 2010). A second possibility is that DNA damage in lymphocytes may be a result of the influence of tumour-associated factors (Smith et al., 2003). Moreover, a deficiency of DNA repair may lead to a higher level of DNA damage.

Evidence from epidemiology, biology, genetic and experimental studies now suggests an association between chronic inflammation and the initiation or progression of various cancers, including prostate cancer (Balkwill and Mantovani, 2001; Coussens and Werb, 2002; Mantovani et al., 2008; Thapa and Ghosh, 2015). The inflammatory cells, cytokines and chemokines all offer a microenvironment that encourages tumour growth by promoting the production of reactive oxygen species. This, in turn, causes oxidative DNA damage and deficient DNA repair capacity and allows tumour progression by promoting angiogenesis (Balkwill and Mantovani, 2001; Coussens and Werb, 2002; Mantovani et al., 2008).

Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin and ibuprofen, are widely used as effective anti-inflammatory, antipyretic and analgesic drugs, and accumulating evidence now suggests that long-term use of NSAIDs can be considered an effective approach for cancer chemoprevention (Baron, 2003; Corley et al., 2003; Holick et al., 2003; Mahmud et al., 2004; Palapattu et al., 2005; Wang et al., 2003).

Our work was based on the induction of DNA damage in lymphocytes treated with bleomycin (BLM). All cells have capacity to repair DNA damage, and bleomycin has been used to induce single and double-strand DNA breaks. In the present study, we used bleomycin to challenge the cells, and then eliminated the bleomycin and treated the cells with nanoformulated and bulk forms of aspirin and ibuprofen to test the effects of NSAIDs on DNA repair capacities (Wei et al., 2005; Zheng et al., 2003).

Treatment of lymphocytes from healthy participants and prostate cancer patients with bleomycin resulted in a significant reduction in DNA repair capacity and increase DNA damage, but ASP and IBU treatment prevented this reduction, with nanoformulated aspirin giving the best response figure 4.1 (A&B) and (C&D). This finding could indicate that aspirin is a stronger antioxidant than ibuprofen, in agreement with previous results showing that aspirin can stabilise DNA and prevent DNA strand breaks induced by oxidative stress (Chen et al., 2009; Hsu and Li, 2002).

We confirmed this finding by measuring ROS levels following T-BHP-induced oxidative stress in lymphocytes from healthy control and prostate cancer patients, and we also investigated the potential protective effect of both forms of aspirin. T-BHP is a well-documented oxidative stress inducer which has been commonly

used in oxidative stress experiments (Wang et al., 2015). Our results showed that lymphocytes from prostate cancer patients generated greater levels of ROS than did lymphocytes from healthy donors, in agreement with previous studies by Toyokuni et al. (1995) and by Kawanishi et al. (2006). Our results also indicated that treatment of lymphocytes with aspirin, in nanoformulated or bulk form, effectively protected the lymphocytes against T-BHP-induced ROS (Figure 4.4).

Moreover, these protective effects significantly decreased the production of T-BHP-induced ROS by these lymphocytes. The nanoformulated aspirin had a more pronounced effect than the bulk form in reducing the ROS, although the difference was not statistically significant.

Human lymphocytes are now believed to metabolise T-BHP by a pathway that includes cytochrome P-450 and cyclooxygenases and results in the generation of toxic peroxyl and alkoxy radicals (Rush et al., 1985). This metabolic pathway could increase the levels of cellular free radicals that would then attack proteins, phospholipids and nucleic acids (Yen and Hung, 2000). Therefore, aspirin may exert its antioxidant activity by inhibiting this free radical generation.

Aspirin is also known to work by inhibiting the activities of cyclooxygenase enzymes. Another possibility is that the effect of aspirin reflects its effects on the expression of inflammatory mediators. Regardless of the precise mechanism, aspirin appears to protect lymphocytes from oxidative stress (Reuter et al., 2010), in agreement with the antioxidant role previously described for aspirin.

# Chapter 5

# Effects of anti-inflammatory drugs on major signal transduction pathways in isolated lymphocyte cells.

## 5.1 Introduction

Effective cancer chemoprevention can involve the use of specific natural, synthetic or chemical agents to restrict, slow the progression of or inhibit carcinogenesis, thus preventing the sequence of biological events leading to the development of invasive cancer. Epidemiological and clinical studies and animal models are supported by evidence from several investigators demonstrating that non-steroidal anti-inflammatory drugs (NSAIDs) and inhibitors of cyclooxygenase 2 (COX-2) may protect against the development of prostate cancer (Norrish et al., 1998; Thun et al., 1993) and other tumours, including colorectal (Janne and Mayer, 2000), breast (Harris et al., 1999; Thun et al., 1993), lung (Muscat et al., 2003; Thun et al., 1993), bladder (Thun et al., 1993), ovarian (Cramer et al., 1998; Thun et al., 1993), oesophageal and stomach cancers (Thun et al., 1993).

The unique pharmacological activities of NSAIDs are attributed to their inhibition of COXs in several tissues and cell types. The inhibition of COX-2 is thought to underlie their chemopreventive properties, most likely via the induction of tumour cell apoptosis (Rao and Reddy, 2004) and the inhibition of tumour cell proliferation and angiogenesis (Abdelrahim and Safe, 2005).

NSAIDs inhibit cell proliferation and change cell cycle progression and can induce apoptosis in prostate cancer lines (Narayanan et al., 2006). Amongst the most widely studied agents showing inhibitory effects on the expression of the COX-2 compounds are aspirin and ibuprofen. Aspirin has been shown to have a chemopreventive effect on prostate cancer via the contribution of COX-independent pathways; for instance, it has been found to inhibit the nuclear transcription factor kappa B (NF-κB) (Wang et al., 2010).

Along a similar line, previous research by Goel et al. (2003) has supported the conclusion that aspirin changes the expression of genes involved in mismatch repair (MMR) and cell cycle control. Previous experimental evidence has indicated the involvement of aspirin in cell cycle progression, although it is not clear how NSAIDs are involved in the biochemical pathways.

Eukaryotic cells employ control mechanisms, termed ‘cell cycle checkpoints,’ that induce cell cycle arrest at specific stages, allowing for the repair of DNA damage to maintain genomic integrity and chromosome stability (Ciccia and Elledge, 2010; Hartwell and Weinert, 1989). Sensors, transducers and effectors make up the DNA damage response pathway.

The cell cycle checkpoint pathway is a signal transduction pathway made up of damage sensors, signal transducers and effectors. Ataxia-telangiectasia-mutated kinase (ATM) and ATM and Rad3-related kinase (ATR) are phosphatidylinositol-3-like kinases essential for sensing DNA damage and activating DNA damage checkpoints, DNA repair and apoptosis (Sarkaria et al., 1999).

ATM, predominantly a nuclear protein, has been identified as the product of a gene that is mutated in a rare multisystem disorder, ataxia-telangiectasia (AT). AT disorder disrupts the normal production of ATM and is characterised by cerebellar degeneration, mostly via the loss of Purkinje and granule cells in the cerebellum; immunodeficiency; cell cycle checkpoint defects and cancer susceptibility (Savitsky et al., 1995).

The main pathway of ATM is initiated and activated by double-strand breaks (DSBs) and can possibly be stimulated throughout all cell cycle phases. Defects in the cell cycle checkpoint mechanism and premature progression during the cell

cycle may be lethal to the cells or cause oncogenic transformation (Savitsky et al., 1995). The key hallmark of malignant transformation in cells is a loss of normal cell checkpoint control, leading to the accumulation of mutations and additional genetic abnormalities (Broustas and Lieberman, 2014).

Once checkpoint arrest control is compromised, induction of the S phase, or mitosis, can take place despite the cellular damage and result in genetic instability, which may contribute to the development of a malignant clone. During this, cells in which checkpoint control is disrupted are most likely subjected to the accumulation of more genetic damage (Zhou and Bartek, 2004).

The tumour suppressor protein p53 plays a key role in the regulation of the cell cycle and is considered the main mediator of checkpoint induced arrest in the G1 phase of the cell cycle. The accumulation and activation of p53 can be initiated by a variety of cellular stress signals that transiently stabilise the protein, including DNA damage, hypoxia, nucleotide deprivation, viral infection, heat shock and mitogenic or oncogenic activation. These lead to the activation of p53 as a transcription factor (Lane et al., 1995).

p53 mediates DNA-damage-induced cell cycle arrest through p21 Waf1, which is known as the inhibitor of several cyclin-dependent kinases (CDKs) and a target for various signals that induce growth arrest and differentiation to prevent DNA replication (el-Deiry et al., 1993).

p53 can also induce apoptosis, predominantly via Bax (Brady and Gil-Gomez, 1998). As many as 50% of all cancers exhibit p53 mutations, and regulation of this protein is defective in a variety of others (Brady and Gil-Gomez, 1998). p53 is stabilised after DNA damage, allowing it to activate the ATM and ATR kinases (Kastan et al., 2000). ATM is required for efficient activation of the cell cycle

checkpoint and homologous recombination following DNA damage (Kastan et al., 2000). Germline ATM mutations lead to increased sensitivity to ionising radiation, immunodeficiency, and a predisposition to cancer (Barzilai et al., 2002). Upon stimulation, ATM undergoes autophosphorylation at Ser1981 and dimer separation (Bakkenist and Kastan, 2003).

As a protein kinase, ATM function by activating DNA repair homologous recombination and checkpoint proteins, including p53 and checkpoint kinase 2 (Chk2). ATM has also been previously shown to activate p53 to induce G1 arrest in the cell cycle following DNA damage (Kang et al., 2005). ATM and ATR both directly stabilize p53 by phosphorylation in vivo on Ser15 and Ser37 (Siliciano et al., 1997). They substrate Chk2 phosphorylates p53 on Ser20, have been shown to play a significant role in regulating the binding to and degradation by Mdm2 and thus in stabilizing p53 (Bartek and Lukas, 2003).

X-ray repair cross-complementing protein 3 (XRCC3) is a member of the RecA/Rad51-related protein family that promotes chromosome stability and is involved in DNA repair. XRCC3 plays a crucial role in the homologous recombination repair (HRR) pathway. Previous studies have shown that direct interaction between XRCC3 and RAD51 may result in their working together in recombination repair mechanisms (Brenneman et al., 2000; Chun et al., 2013; Liu et al., 1998).

Genetic polymorphisms have been found in the XRCC3 gene at locations where some associated genetic modifications may alter DNA repair capacity and therefore be related to increases or decreases in cancer risk.

This chapter will investigate the effects of the nano and bulk forms of two well-known ant-inflammatory drugs, aspirin and ibuprofen, on some key regulatory

signal transduction pathways and DNA-damage-regulating proteins in human prostate lymphocyte cells.

## 5.2 Material and Methods

Please refer to the Chapter 2 Materials and Methods section 2.2.9 for Western blot and section 2.2.10 for qPCR.

## 5.3 Results

### 5.3.1 Analysis of p53 and XRCC3 expressions after *in-vitro* treatment of lymphocytes from healthy individuals and prostate cancer patients with nano-sized and bulk forms of aspirin and ibuprofen

#### 5.3.1.1 Analysis of p53 protein expression after NSAID treatment *in vitro* using a Western blot test

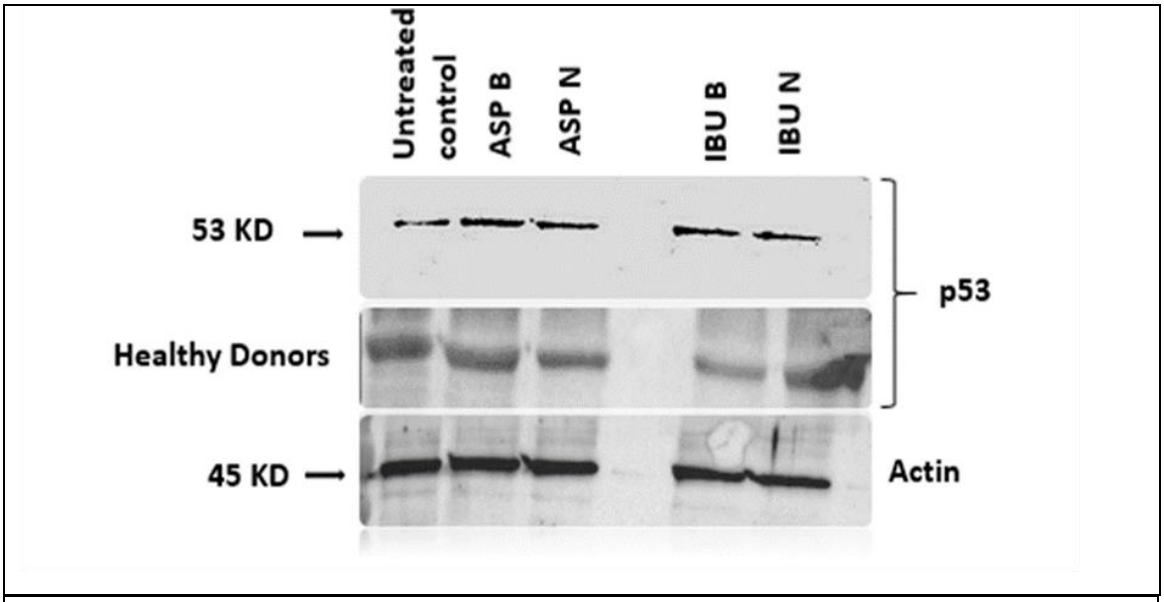
p53, a tumour suppressor protein, plays many roles in several cellular outcomes, such as apoptosis, the ability to induce cell cycle arrest, DNA repair and angiogenesis (Zambetti, 2014). Because apoptosis is an essential process for killing cancer cells, we investigated whether p53 protein levels would be changed in the lymphocytes of prostate cancer patients compared to those of healthy control subjects after treatment with the nano and bulk forms of aspirin and ibuprofen. Western blotting was carried out, and the protein's expression was analysed in lymphocytes from three prostate cancer patients and three healthy individuals and compared to untreated lymphocytes as described in section 2.2.9.10.

Figure 5.1, (B and C) shows that p53 was up-regulated in the lymphocytes of prostate cancer patients after treatment with aspirin bulk (ASP B) 1.6-fold and aspirin nano (ASP N) 1.9-fold compared to the untreated controls.

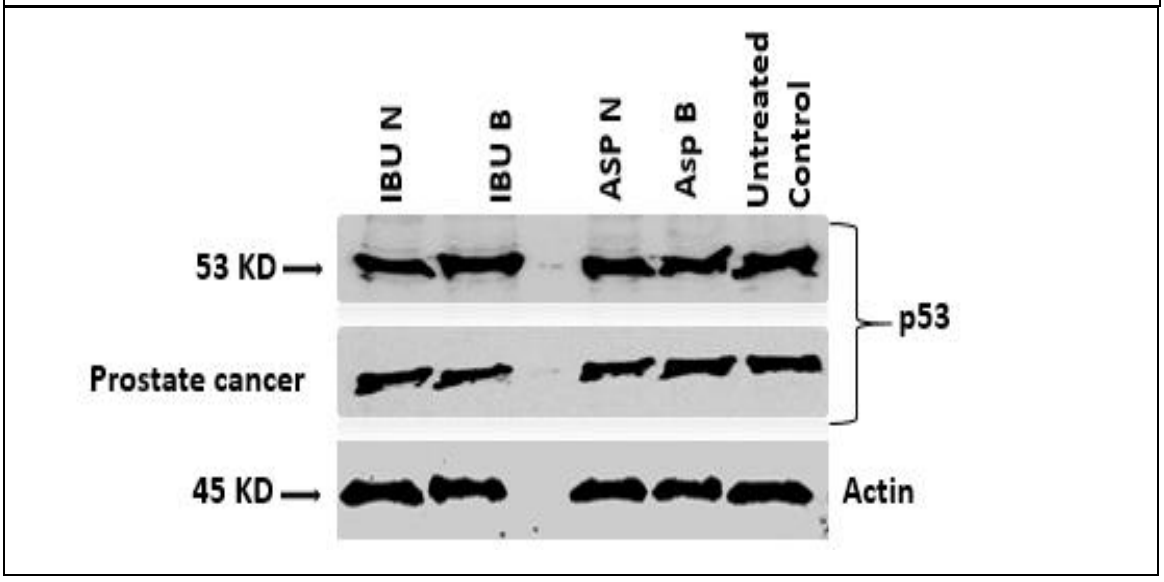
However, p53 expression was reduced in the lymphocyte cells after treatment with ibuprofen bulk (IBU B) and IBU N in both healthy volunteers and prostate cancer patients. Also, p53 showed a 1.3-fold increase in the lymphocytes of

healthy individuals treated with ASP B, a 1.6-fold-increase after treatment with ASP N a 0.67-fold decrease after treatment with IBU B and a 0.66-fold decrease after treatment with ibuprofen nano (IBU N) in healthy volunteers Figure 5, (A and C). Our observation of the increased in vitro expression of p53 in the lymphocyte cells after treated with aspirin indicate that aspirin has apoptotic potential.

**A**




**B**



C

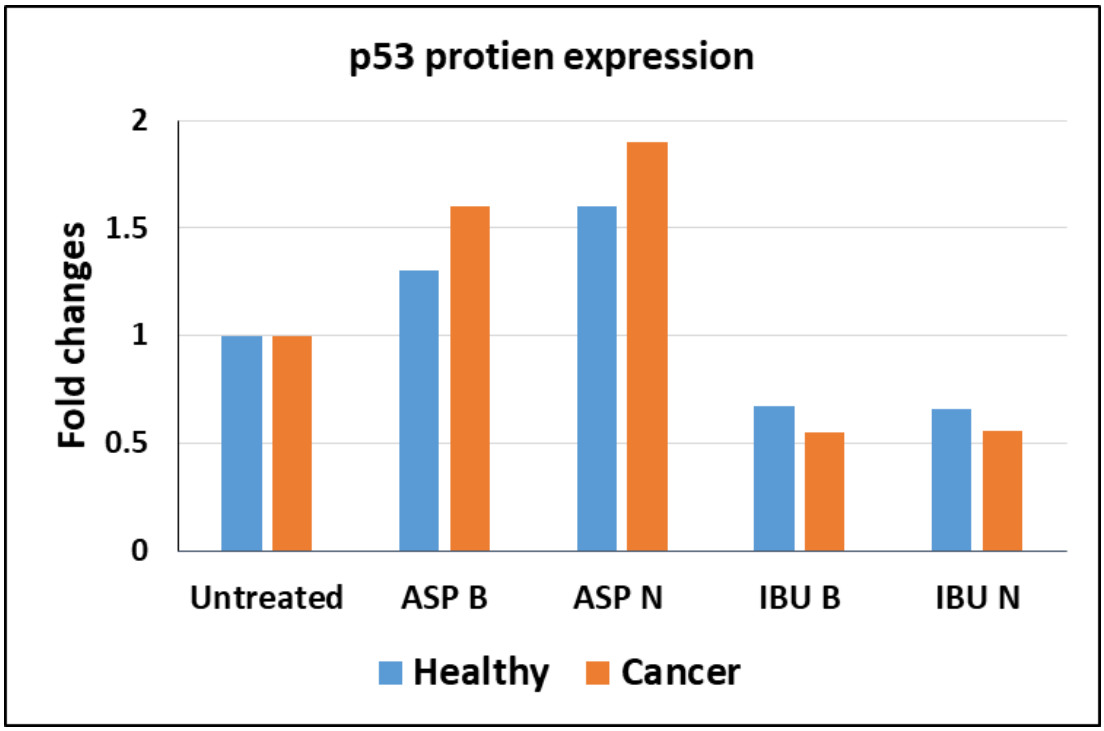



**Figure 5.1. (A, B)** Immunoblot analysis of the p53 protein in healthy volunteers and prostate cancer patients treated with ASP B, ASP N, IBU B, and IBU N. Actin was used as an internal control protein to normalise the data. **(C)** Bar graphs exhibiting fold changes in protein expression levels. Data are represented by the mean ± SEM of three experiments.

### 5.3.1.2 Protein expression analysis of p21 after NSAID treatment

It is well documented that p53 mediates cell cycle arrest in the G1 phase through the transcriptional activation of p21 Waf1/Cip1, an inhibitor of CDKs 2, 3, 4 and 6. Western blot analysis was used to examine the expression of the p21 CDK inhibitor. After 24 hours, the p21 protein level was up-regulated in the ASP B-treated lymphocytes from healthy donors and prostate cancer patients (Figure 5.2, A and B). However, the difference between the increases in the tested protein's expression profile in the prostate cancer patients and the controls was not significant (Figures 5.2). Also, no change in the p21 levels was observed in the lymphocytes of healthy donors or prostate cancer patients on exposure to

ASP N, IBU B or IBU N, indicating the presence of a p53-independent mechanism for p21 Waf1/Cip1 up-regulation in these cells.

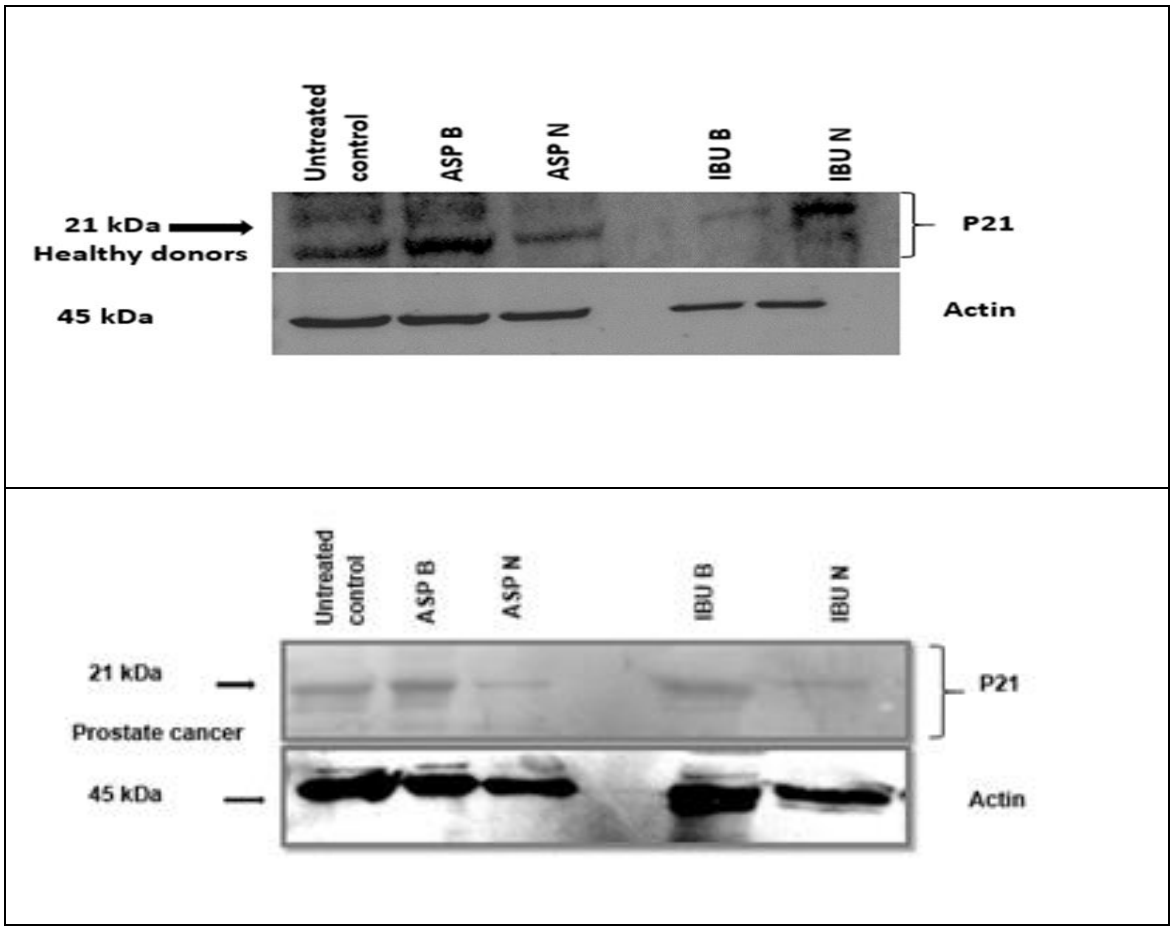


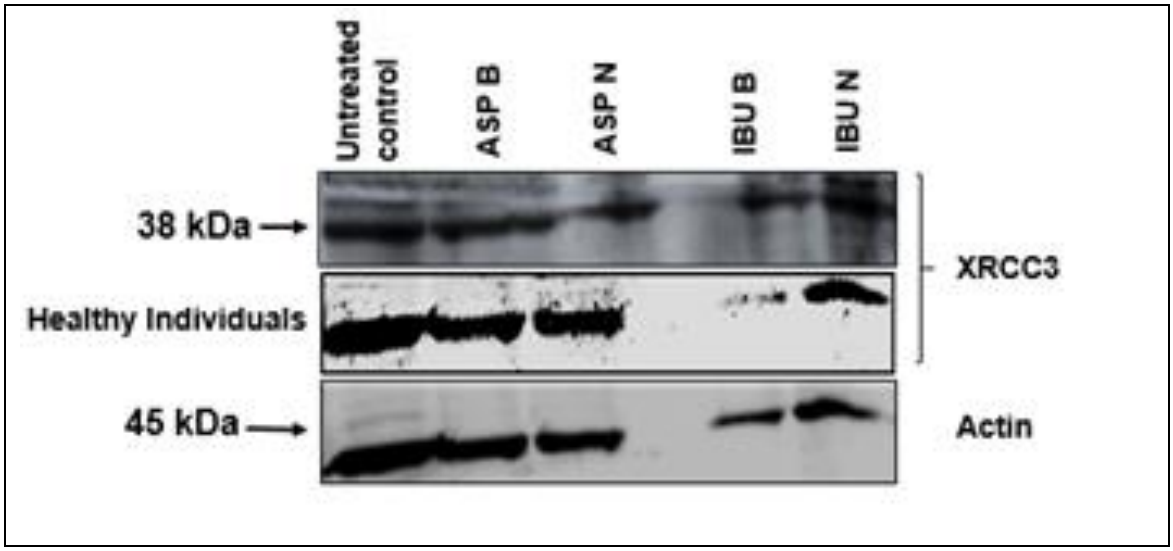

**Figure 5.2. (A, B)** Immunoblot analysis of the p21 protein in healthy volunteers and prostate cancer patients treated with ASP B, ASP N, IBU B and IB N. Actin was used as an internal control protein to normalise the data.

### 5.3.1.3 Evaluation of XRCC3 protein expression in vitro using a western blot test

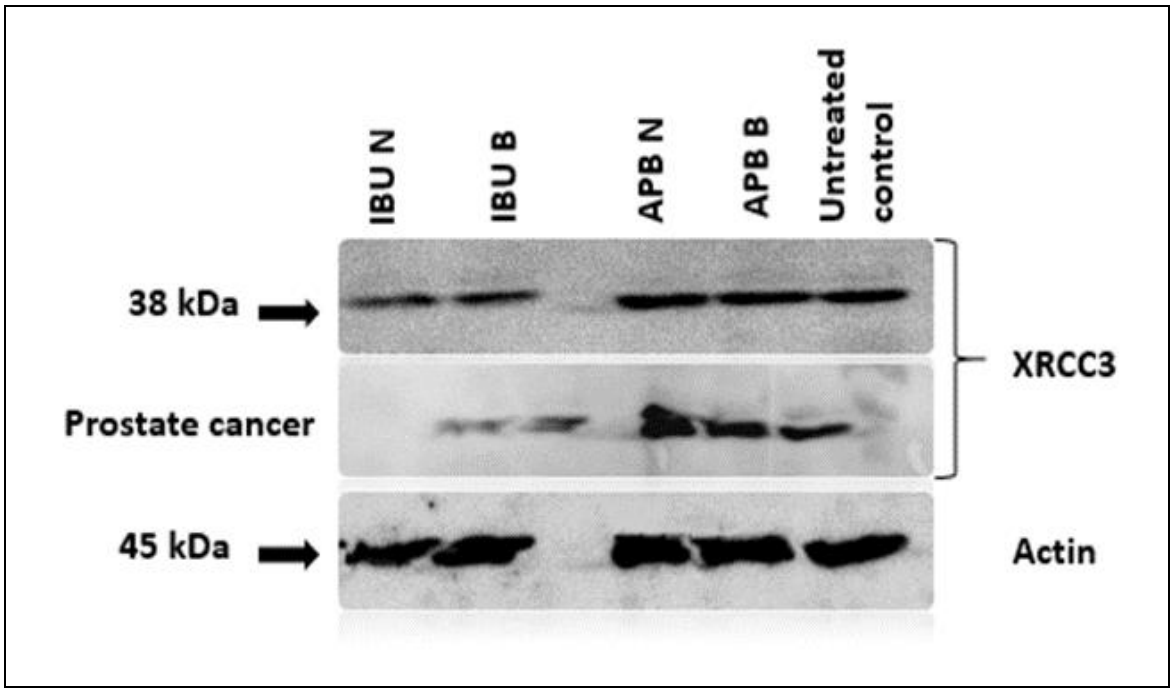
There are two useful mechanisms for repairing double-strand breaks, homologous recombination (HR) and non-homologous end-joining (NHEJ), of which HR is the more precise. The XRCC3 gene encodes a member of the RecA/Rad51-related protein family, known to participate in HR to maintain chromosome stability and repair DNA damage (Thacker and Zdzienicka, 2004).

XRCC3 was therefore chosen for investigation in the present study. The western blot test was used to evaluate XRCC3 protein expression in lymphocytes from prostate cancer patients and healthy volunteers who were treated with 500µg of the nano and bulk forms of aspirin and ibuprofen. Untreated cells were used as controls to determine the cut-off value of XRCC3 overexpression. As shown in Figure 5.3, A, B and C, the level of XRCC3 expression was notably increased in the lymphocytes from both prostate cancer patients and healthy donors after 24-hour exposure to both the nano and bulk forms of aspirin. However, the expression of XRCC3 was reduced after treatment with both forms of ibuprofen. In both cases, there was no statistical difference between the XRCC3 expression levels of untreated lymphocytes and of those treated with aspirin or ibuprofen.

**A**



B




C

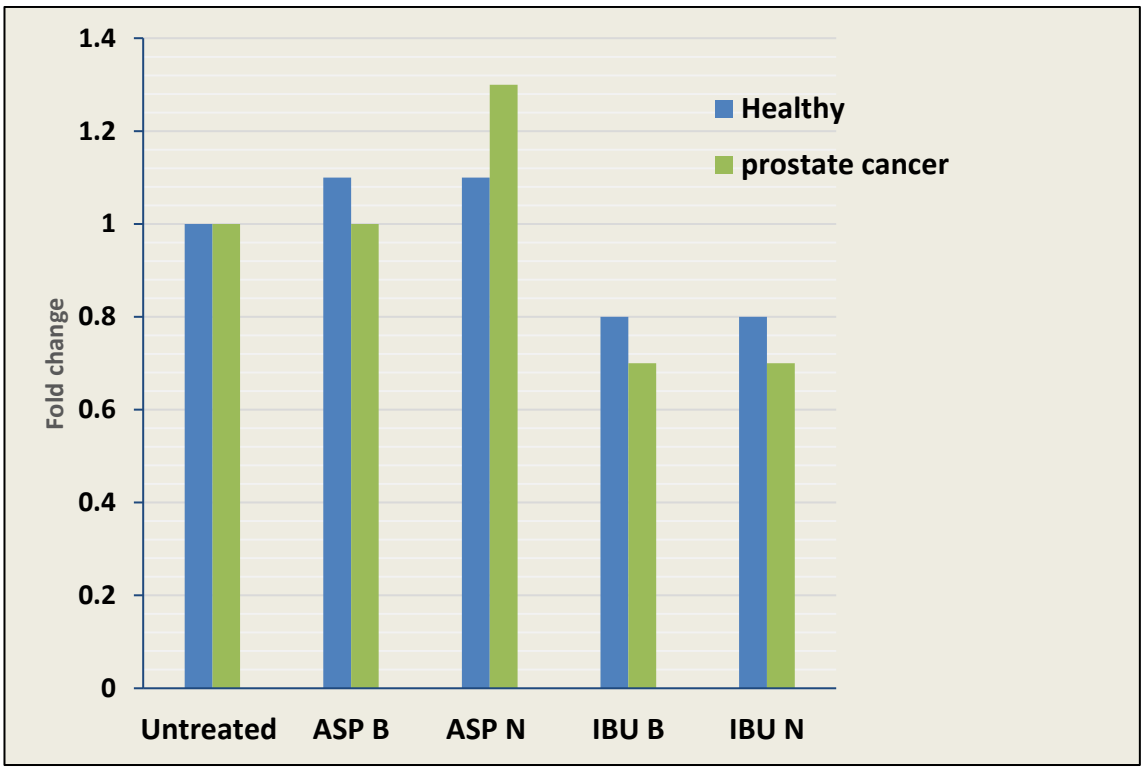



**Figure 5.3. (A, B)** Immunoblot analysis demonstrating changes in XRCC3 expression after 24-h treatment with both forms of aspirin and ibuprofen compared with untreated lymphocyte samples. Actin was used as a loading control. **(C)** Bar graphs exhibiting fold changes in the protein expression levels. Data are represented by the mean ± SEM of three experiments (n=3 for each treatment).

### 5.3.2 NSAIDs activate the ATM and ATR signalling pathway independent of DNA damage in lymphocyte cells.

Based on our previous results, we concluded that NSAIDs reduce DNA damage in lymphocytes. We further investigated the effects of both forms of aspirin and ibuprofen on the gene expression of ATM and ATR in lymphocyte cells. Total RNA was isolated from lymphocyte cells that had been treated for 24 hours with both forms of aspirin and ibuprofen and was subjected to quantitative RT-PCR. The results were consistent with the induction of significant increases in p53 and p21 protein levels in lymphocytes within 24 hours after treatment with ASP N and ASP B, respectively (Figures 5.1 and 5.2, A and B).

The real-time PCR results are shown in Figure 5.4 and indicate that all examined gene expressions associated with DNA damage and repair, such as that of ATM mRNA, were significantly increased after treatment with ASP B, ASP N and IBU N. However, the best result was obtained with ASP N (Figure 5.4). ATR expression was also increased significantly with IBU N. This suggests that NSAIDs do not activate ATM and ATR through the induction of DNA double-strand breaks (Figure 5.4, 5.5).

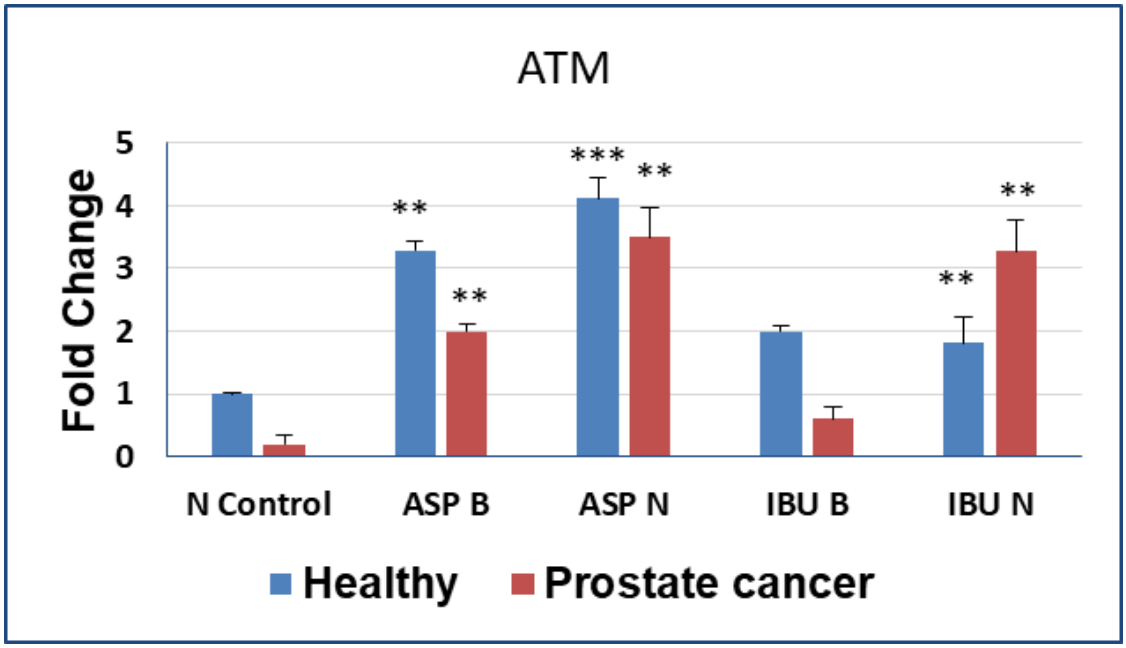



**Figure 5.4** The influence of aspirin and ibuprofen, nano-sized and bulk form, on the expression of ATM mRNA in lymphocyte cells. β-actin was used as an internal control gene. ATM gene expression analysis was performed on lymphocytes from healthy individuals (blue bar) and prostate cancer patients (red bar) after 24-hour treatment. Values are the means of three independent experiments, and the error bars represent SDs. The p values are * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

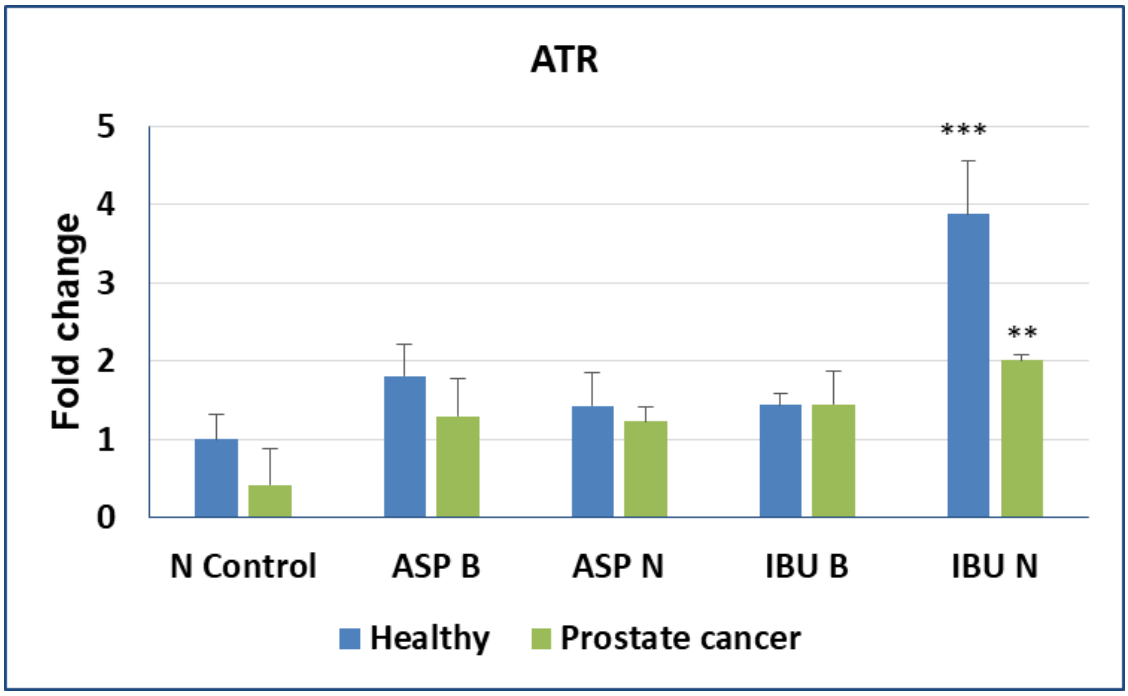



**Figure 5.5** ATR expression in lymphocyte cells after 24 hours of exposure to aspirin and ibuprofen, nano and bulk forms. The fold changes in ATR gene expression using qRT-PCR are shown. Results represent three experiments. Values are the means of three independent experiments, and the error bars represent SDs. The p values are * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

## 5.4 Discussion

Anti-inflammatory drugs have attracted considerable attention as one of several drug types that may exhibit cancer chemopreventive activity, in particular for prostate cancer (Narayanan et al., 2004). The current interest in these drugs is based on observations from epidemiological, clinical, and animal studies in which they have shown antineoplastic effects (Giovannucci et al., 1995; Greenberg et al., 1993; Thun et al., 2002).

The exact mechanisms by which these drugs inhibit tumour formation and growth remain unclear. One proposed mechanism is their direct inhibition of COX in prostate cells, as prostaglandin levels are enhanced in prostate cancer (Wang et al., 2011). Moreover, COX-2 overexpression in epithelial cells contributes to the inhibition of apoptosis and proliferation (Sobolewski et al., 2010).

However, the chemopreventative effects of NSAIDs are not based exclusively on their COX inhibition, as NSAIDs can trigger cell death by apoptosis in cells that lack detectable COX-2 activity (Yu et al., 2002). The genetic predisposition to cancer and hyper-sensitivity to ionising radiation detected in AT patients can be associated with chromosomal instability, abnormalities in genetic recombination and failure to activate cell signalling for programmed cell death and cell cycle checkpoints in response to DNA-damage agents. ATM mutations are involved in the development of sporadic human cancers such as leukemia (Stilgenbauer et al., 1997).

The loss of heterozygosity at the ATM gene locus has also been reported in many cancers, including metastatic prostate carcinoma (Angèle et al., 2004; Ruijter et al., 1999). These prior studies suggested that the ATM gene encodes a protein that senses damage and transduces signals into cells for promoting apoptosis

(Marinoglou, 2012; Shiloh, 1997). Recent evidence also points to the possibility that ATM proteins may stimulate effective defence mechanisms against oxidative injury (Barzilai et al., 2002; Semlitsch et al., 2011; Watters, 2003).

Our results show that the activation of ATM-dependent checkpoints occurs independently of aspirin's anti-inflammatory properties. Our study's indications that ATM participates in the aspirin-mediated checkpoint response include the auto-phosphorylation of ATM and its targets, p53 and p21 (Figure 5.4, 5.1 and 5.2), as well as ATM's effectiveness at controlling cell cycle arrest and apoptosis (Figure 5.1, C). This indicates that ATM's effect on cell cycle arrest and apoptosis is one of the study's indications that it participates in the checkpoint response mediated by aspirin.

Some oncogenes and specific tumour suppressor genes play a crucial role in human prostate cancer carcinogenesis (Isaacs and Kainu, 2001; Turner and Watson, 2008). Most cancer cells have a defective tumor suppressor gene for p53, rendering this protein incapable of controlling cell proliferation and resulting in ineffective DNA repair. The most frequent genetic changes to p53 in cancer tissues are missense mutations, which result in a 'loss-of-function' phenotype. This type of alteration forms in numerous cancers, including prostate cancer (Muller and Vousden, 2014; Supek et al., 2014).

Recent studies have shown that small-molecule drugs may activate p53 to suppress cancer cell proliferation via cell cycle arrest or apoptosis. This opens up new possibilities for fighting and preventing cancer (Fuster et al., 2007). It is well documented that p53-inducible pro-apoptotic genes trigger apoptosis through both the extrinsic and intrinsic apoptotic molecular pathway (Kuribayashi and El-Deiry, 2008). The stabilisation of p53 has also been found to mediate

apoptosis, as shown after treatment with aspirin (Goel et al., 2003). P53 phosphorylation and stabilisation by non-genotoxic stress have both been well documented in previous studies. For instance, treating colon or lung cancer cells with taxol or nocodazole leads to the stabilisation and phosphorylation of p53 at serines 6, 15, 33, 46 and 392 (Saito et al., 2003). However, these studies did not explore the mechanism of ATM signalling in human prostate carcinoma cells. Our results show that 24-hour treatment with both the nano and bulk forms of aspirin activates and stabilises the p53 protein at the post-translational level (Figure 5.1, C), suggesting that aspirin may ensure p53-dependent action—for instance, cell cycle arrest and apoptosis.

Treating lymphocytes from prostate cancer patients and healthy volunteers with both forms of aspirin and ibuprofen resulted in p53-mediated cell cycle arrest and apoptosis.

The CDK inhibitor p21 is a key transcriptional target of p53 and mediates cell cycle arrest in G1 and G2. In our study, we indicated the CDK inhibitor p21 as the critical mediator for an aspirin-dependent G1/S checkpoint. A previous study by Hardwick et al.(2004) showed that p21 was up-regulated at both the gene and protein levels in HT29 cells treated with 5-mM aspirin. Consistent with this finding, the present study also revealed a significant increase in the expression of p21 after treatment with aspirin (Figure 5.2, A and B), suggesting that the nano-sized aspirin induced the inhibition of cell proliferation via cell cycle arrest.

ATM activation has been shown after treatment with genistein (isoflavonoid), in which the ATM-dependent phosphorylation of p53 on Ser15 stimulates p21 expression (Chang et al., 2004). However, genistein is known as a possible DNA-damaging agent because it inhibits topoisomerase II by stabilisation of the DNA

cleavage complex, an event predicted to cause DNA damage (Ye et al., 2001). Another study showed that Indole-3-carbinol activates the ATM signalling pathway independent of DNA damage to stabilize p53 and induce G1 arrest of human mammary epithelial cells (Brew et al., 2006).

In our previous results in chapter 3, the Comet assay showed that aspirin did not induce DNA damage. Thus, our study is the first account of aspirin signalling through the ATM and ATR pathway without inducing DNA damage. Our results suggest an ATM-dependent mechanism of action for aspirin in the chemoprevention of prostate cancer. Aspirin is mostly effective via the p21 pathway, as its antiproliferative activity is p53 dependent.

The main effect of ATM activation is the up-regulation of p21. It has been indicated that ATM is recruited to the site of DNA damage, and it can directly bind to and phosphorylate proteins involved in DNA repair, such as c-Abl, Brca1, Nbs1 and replication protein A (Jimenez et al., 1999). This suggests that by activating the G1/S checkpoint, aspirin increases opportunities for lymphocyte cells to repair DNA damage before replication or to induce apoptosis, both of which may contribute to maintaining the integrity of genomic DNA.

Sensor protein complexes scan the DNA for abnormalities and translate into activating signals for downstream target proteins, such as the ATM kinase. Despite numerous studies on the molecular components of checkpoints, however, both the identities of these sensors and their mechanisms of action remain unclear.

It seems possible that aspirin interferes with such sensors upstream of ATM. The identification of additional aspirin targets aside from COX-2 will help in the

understanding of its antitumor effects and in the design of novel chemopreventive agents.

XRCC3 is a Rad51 paralog and is involved in homologous recombination and the repair of DNA double-strand breaks. Our results show that XRCC3 protein expression, measured by immunoblotting analysis, is increased in lymphocytes after 24-hour aspirin treatment compared to its expression in untreated cells (Figure 5.3). This result is consistent with a previous finding indicating that XRCC3 depletion by siRNA in MCF7 cells could inhibit cell proliferation, cause increased DNA damage and promote p53-dependent cell death (Loignon et al., 2007).

In contrast, a previous study investigating XRCC3 expression in the breast cell line found that aspirin can induce the overexpression of XRCC3-induced cisplatin resistance (Xu et al., 2005).

# Chapter 6
# General Discussion

## 6.1 Discussion

The human body contains approximately $10^{13}$ cells, and each of them is calculated to receive tens of thousands amount of DNA damage insults per day (Lindahl and Barnes, 2000). The resulting lesions can block genome replication and transcription, and if unrepaired or incorrectly repaired, these lesions lead to mutations that threaten cell or organism viability and finally may cause genome instability and cancer (Lindahl and Barnes, 2000). Consequently, evaluation of genotoxicity mechanisms has become an important part of drug validation processes. The molecular genetic mechanisms of aspirin and ibuprofen nano and bulk forms are not fully understood, especially in lymphocyte cells and these mechanisms therefore warrant additional study. The comet assay and micronucleus assay have been accepted as a valid test for genotoxicity by the regulatory agencies due to their sensitivity and high statistical power in identifying mutagenicity in the form of the DNA and chromosomal damage. As this work is exploratory, the in vitro comet and micronucleus assays were used as recommended for NPs (Magdolenova et al., 2014).

The probable causative linkage that is best established in humans is the relation between chronic inflammation-induced DNA damage and the incidence of cancer. Strong links exist between the presence and persistence of inflammation at the particular site where pre-cancerous lesions develop (Grivennikov et al., 2010), suggesting that inflammation promotes cancer development and progression. The hallmarks of chronic inflammation include up-regulation of pro-inflammatory mediators and dynamic changes in the microenvironment that promote the malignant transformation of cells and carcinogenesis via several events, including enhanced incidences of DNA damage, enhanced cellular

proliferation and inhibition of apoptosis (Hofseth and Ying, 2006). A role for inflammation in tumour generation is now generally accepted, and inflammation is now a vital target in cancer prevention and treatment.

The present studies investigated the effect of two well-known anti-inflammatory drugs, aspirin and ibuprofen, on DNA damage in peripheral lymphocytes from healthy volunteers and from patients with prostate cancer to determine whether these drugs have genoprotective or genotoxic effects on lymphocyte cells (Chapter 3).

Our comet assay results indicate that no DNA damage occurred in either study group treated with aspirin or ibuprofen in either the bulk (ASP B, IBU B) or nano (ASP N, IBU N) forms. In fact, ASP N treatment significantly decreased the DNA damage (figures 3.3, 3.4, 3.5, 3.6 and tables 3.2, 3.3), indicating a possible genoprotective effect of this formulation. NSAIDs act by inhibiting COX activity, thereby reducing the effects of inflammation, so this is now a fundamental drug class being investigated to discover factors to improve the outcome of several cancerous states, beyond acting as a preventative measure and a standard (Brune and Patrignani, 2015).

The CBMN assay was used to investigate the formation of micronuclei generated by treatment with aspirin and ibuprofen in both the bulk and nano forms. The micronucleus frequency was significantly increased in patients with prostate cancer when compared to healthy individuals (figure 3.8 and table 3.7). The prostate cancer group, following treatment with ASP N and ASP B, showed a significant decrease in the frequency of micronuclei, with ASP N showing significantly different levels when compared to ASP B. However, the frequency of micronuclei was increased after treatment with IBU B and IBU N in both groups,

and the frequency of micronuclei was higher for IBU B than for IBU N (figure 3.8 and table 3.7). The nuclear division index ranged from 1.74–1.92 for healthy donors and from 1.70–2.00 for patients with prostate cancer (table 3.7), which fell within the normal range of 1.3–2.2 (Fenech, 2007). These results indicate that a decrease in drug particle size is associated with the surface atom ratio increase, and the reactivity also increases. The comet and micronucleus assay results agree with the findings of Najafzadeh et al. (2016) in which aspirin showed an anticancer effect in lung cancer. Several experimental and clinical studies have also demonstrated the chemo-preventive potential of aspirin use in cancer prevention in association with inhibition of metastasis and reduction in cancer incidence and mortality in colorectal, adenocarcinoma, prostate, lung, pancreatic, breast and oesophageal cancers (Bosetti et al., 2012; Rothwell et al., 2012; Streicher et al., 2014). All these previous studies, however, contradict the results of studies that indicate no relationship between aspirin use and a reduction in cancer incidence (Cook et al., 2005; Gann et al., 1993). Similarly, no association has been indicated between aspirin use and an increased incidence of breast cancer (Friis et al., 2008; Johnson et al., 2002).

The comet assay findings prompted a further study to investigate DNA repair capacity using a challenge assay. The basic purpose of challenge assays is to expose cells to a DNA damaging agent and then measure how the subsequent DNA repair of cells responds in the presence and absence of NSAID. The aim here was to determine whether particle size (nano vs bulk) affected the ability of aspirin and ibuprofen to facilitate DNA repair in lymphocytes from patients with prostate cancer and healthy volunteers.

Previous evidence showed that capacity for DNA repair is one of the important determinants of susceptibility to cancer (Gaivao et al., 2009). Lymphocytes were challenged with BLM and then the BLM was removed to allow DNA repair. Some DNA repair was tested in the presence of NSAIDs, as previously discussed. The average background DNA damage in lymphocytes was higher in the untreated prostate cancer group than in the healthy donor's figures 4.1–4.2 (A, B and C, D). Following exposure to BLM, the repair was slower in lymphocytes from the prostate cancer group than from the healthy volunteers (figure 4.3). These differences could have arisen due to changing genetic backgrounds, such as polymorphisms in the genes coding for repair. A previous study conducted by; Cheng et al. (2002) showed that polymorphism and the level of expression of nucleotide excision repair genes was related to an increased risk of head and neck cancers. Differences in the efficiency of nucleotide excision repair could influence the repair of oxidative damage induced by irradiation. Following exposure of lymphocytes to BLM, we examined the protective effects of NSAIDs on the BLM-induced genotoxicity. Figures 4.1 and 4.2 show that DNA damage decreased in cells treated with both forms of aspirin and ibuprofen. However, the nano-sized aspirin showed the best reduction of DNA damage when compared to cells treated with BLM only. The genotoxicity of BLM is thought to mainly reflect the excessive production of ROS. Therefore, treating cells with aspirin may reduce BLM-induced oxidative stress through the radical scavenging activity of the aspirin.

Evidence also suggests that aspirin may increase the capacity of DNA repair mechanisms (Dibra et al., 2010). The X-ray repair cross-complementing protein 3 (XRCC3) is essential for maintaining chromosomal stability and repairing DNA

damage (Brenneman et al., 2000). Genetic polymorphisms have been found in the XRCC3 gene at locations where some associated genetic modifications may alter DNA repair capacity. This then could be related to increases or decreases in cancer risk. Therefore, XRCC3 protein expression was determined by western blots of nuclear lymphocyte extracts (figure 5.3, A, B and C). This is the first report, to the author's knowledge, to examine a role for aspirin and ibuprofen in nano and bulk form on the expression of the XRCC3 protein. As shown in (figure 5.3, A, B and C), the level of XRCC3 expression was notably increased in the lymphocytes from both prostate cancer patients and healthy donors after a 24 h exposure to both the nano and bulk forms of aspirin. However, the expression of XRCC3 was reduced after treatment with both forms of ibuprofen, indicating that only aspirin is involved in the DNA repair.

Chronic inflammation is associated with oxidative stress and free radical production, which can damage genomic DNA and enhance the development of prostate carcinogenesis, particularly in the presence of androgens (Ames and Wakimoto, 2002). The relationship between the nanoparticle-induced ROS production and toxicity is well documented (Fu et al., 2014). The literature has described the role of aspirin as an antioxidant and how it promotes resistance of endothelial cells to oxidant injury (Grosser et al., 2003). However, another study showed that aspirin showed a poor antioxidant effect (Guerrero et al., 2004).

No reports are available that describe a role for the antioxidant effects of aspirin (either bulk or nano-sized) in the protection of lymphocytes from patients with prostate cancer and healthy donors against cell death caused by ROS. Accordingly, we investigated whether ASP N would overcome an oxidising environment or alternatively reduce some of the DNA damage generated in the

presence of TBHP by ROS. ASP N reduced ROS formation and was the most effective at inhibiting ROS generation (figure 4.4), suggesting that ASP N does not induce ROS and has antioxidant properties. The antioxidant activity of aspirin has been implicated as a potential mechanism to explain the cancer preventative effects of aspirin. It is considered an antioxidant with the ability to scavenge *OH radicals, as demonstrated in vitro (Shi et al., 1999). Ferritin appears to be a molecular target of aspirin with a particular potential for clinical significance. Moreover, ferritin induction by aspirin might explain earlier observations showing that aspirin is capable of directly protecting cultured lymphocytes from oxidant injury. The results of the present investigation suggest that, by increasing the synthesis of iron-scavenging ferritin, aspirin may specifically remove iron ions from the site of oxygen radical formation. Aspirin may therefore effectively interrupt the reaction cascade that leads to oxidative stress and tissue damage, thereby explaining its protective action and demonstrating an antioxidant function of newly synthesised ferritin under these conditions.

The mechanism of action of the NSAIDs was further examined by exploring how the NSAIDs exert their control on apoptosis. The tumour suppressor protein, p53, is involved in several cellular outcomes, such as apoptosis and angiogenesis. Therefore, some proteins associated with p53 were examined by Western blotting to clarify whether aspirin and ibuprofen (nano and bulk versions) affect the viability of lymphocyte cells through actions on the p53 protein and effects on p21. We found that ASP N up-regulated expression of the p53 and p21 proteins. The data indicate that aspirin (nano and bulk) induced p53 mediated cell cycle arrest and apoptosis in lymphocytes through stabilisation of the p53 protein. These findings provide a new insight into the cancer chemoprevention properties

of anti-inflammatory drugs (figure 5.1,A and B) via effects on cell cycle progression that activates cellular checkpoints in lymphocytes.

Gene expressions of ATM and ATR were also evaluated by quantifying the level of mRNA using qRT-PCR in order to determine the possible effects of treatment of lymphocytes with both bulk and nano forms of aspirin and ibuprofen. The results show for the first time that treatment of lymphocytes with both forms of aspirin and ibuprofen significantly increased the levels of ATM mRNA; however, the highest expression was obtained with ASP N (figure 5.4). ATR expression was also increased significantly with IBU N (figure 5.5). These findings suggest that NSAIDs do not activate ATM and ATR through the induction of DNA double-strand breaks.

Aspirin can also affect mitochondrial functions. It increases the mitochondrial membrane permeability, causing the release of cytochrome c, resulting in the activation of caspases followed by cell apoptosis in several cell lines (Dikshit et al., 2006).

The electron transport reactions involving cytochrome c are as follows:

$Fe^{2}$+-cytochrome c + 8 $H^{+}_{in}$ + $O_2$ → 4 $Fe^{3+}$-cytochrome c +2 $H_2O$ + 4 $H^{+}_{out}$ Two electrons are delivered from two molecules of cytochrome c in the CuA and cytochrome a sites to the cytochrome a3- CuB binuclear centre, reducing the metals to the $Fe^{2+}$ and $Cu^{+}$ forms. The hydroxide ligand is protonated and removed as water, producing a void in the middle of the metals that is filled by $O_2$. The oxygen is quickly reduced, with two electrons arriving from the $Fe^{2+}$cytochrome a3, which is changed to the ferryl oxo form ($Fe^{4+}$=O). The oxygen atom close to CuB picks up one electron from $Cu^{+}$, and a second electron and a proton from the hydroxyl of Tyr (244), which is converted into a tyrosyl radical:

the second oxygen is converted to a hydroxide ion by picking up two electrons and a proton. A third electron from another cytochrome c is passed through the first two electron carriers to the cytochrome a3- CuB binuclear centre, and this electron and two protons transform the tyrosyl radical back to Tyr and the hydroxide bound to $CuB^{2+}$ to a water molecule. The fourth electron from another cytochrome c flows through CuA and cytochrome a to the cytochrome a3- CuB binuclear centre, reducing the $Fe^{4+}$=O to $Fe^{3+}$, with the oxygen atom picking up a proton and regenerating this oxygen as a hydroxide ion coordinated in the middle of the cytochrome a3- CuB centre, as it was at the start of this cycle. The net process is that four molecules of reduced cytochrome c are used, along with four protons, to reduce $O_2$ to two water molecules.

Aspirin and ibuprofen in their existing forms have drawbacks in their bioavailability. In biopharmaceutics classification system (BCS), aspirin is a class III drug and therefore its rate of dissolution and rate of diffusion (absorption) are its limiting steps. Ibuprofen is a BCS class II drug, so it is only slightly soluble in water; poor solubility can lead to low bioavailability and therefore bioavailability is limited by its dissolution rate. Nano-suspension forms can improve the solubility and increase the bioavailability of both drugs. Our comparison of the nano-formulation of the drugs to their bulk forms demonstrates that a decrease in particle size yields a difference in response. In all cases, the nano form of the drug gave a better response than the bulk form. This highlights the potential of these drugs in their nano form for use as therapeutic options.

Nano-particles have a larger surface area than the bulk forms and the surface atoms are more reactive since, as the surface atoms ratio increases, the reactivity

also increases. At the nano-scale, a huge number of atoms at the surface are exposed. These atoms will strongly participate in any reaction, whereas in bulk material, most of the atoms lie in the interior and can participate only weakly in any reaction (De Jong and Borm, 2008). Nano-particles, because of their small size, can also extravasate through the endothelium in inflammatory sites, epithelia and tumours or can penetrate microcapillaries. In general, the nano-size of these particles allows for efficient uptake by a variety of cell types and selective drug accumulation at target sites (Panyam et al., 2003).

## 6.2 Conclusion

Anti-inflammatory drugs have great potential to be used as adjuvants and in combination therapy in cancer treatment. They thus represent a novel, less toxic treatment option than conventional treatment methods. Unfortunately, the use of these agents is currently restricted due to their side effects. The present study, however, has demonstrated increased activity when particle size is decreased to the nano scale, offering bioavailability without increasing genetic toxicity during in vitro assays on human lymphocytes. It is the researchers' hope that the data presented on the genotoxicity mechanisms of NSAIDs agents in lymphocytes will shed new light on cancer prevention and treatment.

The results demonstrated, via comet and micronucleus assay, that aspirin nano (ASP N) causes a significant decrease in DNA damage compared to aspirin bulk (ASP B) and ibuprofen nano (IBU N). Micronuclei (MNi) decreased after ASP N and ASP B in healthy participants and prostate cancer patients. Gene expression analysis of ATM and ATR suggested that changes in transcript levels of these genes do not necessarily correlate with DNA damage. Instead, XRCC3 expression is up-regulated as protein in the lymphocytes as a result of exposure to aspirin. Aspirin may thus have the capacity to upregulate XRCC3 expression and, by extension, function, which has important implications regarding the response of host cells to chemotherapeutic agents. The significance and utility of this study is further underlined by the finding that aspirin was capable of decreasing reactive oxygen species and DNA strand breaks in human lymphocytes.

## 6.3 Further work

In the literature, no research has yet described a mechanism for aspirin nano-form effects on Bcl, COX1 and COX2 gene expression. Additionally, aspirin has been found to inhibit IkB kinase (IKK) b and prevent NF-kB activation. The only COX independent target that is known to directly interact with aspirin is IKK. Transcription of several proteins involved in inflammatory responses and angiogenesis is promoted by NF-kB; therefore, inhibition of this pathway may also contribute to the observed anticancer effects. Studying the effect of aspirin nano on the protein expression of IKK and NF-kB will be very important as will understanding the effects of aspirin on antioxidant defences against these ROS, such as the glutathione redox cycle and catalase.

# Chapter 7: References

# 7 .References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# Appendix 8

## 8.1 Appendix I


Centre Number:

### 8.1.1 CONSENT FORM FOR PATIENTS

Title of Project: **Effect of small (nano) particles on white blood cells in patients with chest diseases compared to white blood cells in people without chest diseases. (Version 3, 07- 07- 09)**
**Reviewed by Leeds Central Research Ethics Committee (REC) (REC reference number: 12/YH/0464)**

1. I confirm that I have read and understand the information sheet (version 3, 19- 06-09) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights beingaffected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the NHS Trust or the University of Bradford, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
4. I agree that the sample I have given and the information gathered about me can be stored at the University of Bradford, as described in the attached information sheet. ☐
5. I agree to take part in the above study. ☐

| Name of Patient | Date | Signature |
|---|---|---|
| | | |
| Name of Person taking | Date | Signature |
| | | |

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes.

## 8.2 Appendix II


### 8.2.1 Participant Information Sheet for patient

Study title: Genotoxic effects of nano and bulk forms of aspirin and ibuprofen on blood samples from prostate cancer patients compared to those from healthy individuals.

**Reviewed by Leeds Central Research Ethics Committee (REC)**
**(REC reference number: 12/YH/0464)**

**Invitation to the research study**

We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish to and you will be allowed around 24hours to consider this.

(Part 1 tells you the purpose of this study and what will happen to you if you take part.

Part 2 gives you more detailed information about the conduct of the study).

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

**Part 1**

**What is the purpose of the study?**
In this study white blood cells will be treated in a test tube with very small chemical particles to determine if patients with cancerous and inflammatory diseases are more at risk after exposure. A blood sample of around 6-8 teaspoons (40 ml) will be taken. Samples will be stored only for the duration of the study and used for studies of a similar nature or to check original responses. The research is for a PhD programmes involving post-doctoral fellows and PhDs.

**Why have I been invited?**

You have invited because you have disease states and we should like to determine if these small chemical particles could be more harmful to you than to people without diseases state than those without such disease.

**Do I have to take part?**
It is up to you to decide. We shall outline the study and go through this information sheet, which we shall then give to you. We shall ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

**Part 2**
**What will happen to me if I take part?**
A single blood sample will be taken and you will not need to attend the Clinic again for this research study. A brief questionnaire will need to be completed by the researchers.

We shall need to access your notes so that they can be linked in an anonymous way to your clinical data which can be tied up with the research results. Each individual will be given a coded study number.

The data obtained will only be available to the research team and will **not** be returned to you. Responses will be compared only on group basis i.e. collective responses from patients with chest diseases compared to collective responses from people without chest diseases. Results could be published in the form of scientific papers. The work will benefit the medical and scientific community at large, but will not be of direct benefit to you as an individual. If, however, you would like more information, Dr BK Jacobs will be prepared to talk to you individually about study results.

**People who cannot take part in the study.**
People who are not well enough to take part will be excluded (e.g. those with anaemia) If you have any further questions, you could contact the research team: Professor Diana Anderson, Established Chair in Biomedical Sciences, BSc MSc PhD DipEd FSB, FATS, FRC Path, FIFST, FBTS, FRSM, FHEA, FRSC, University of Bradford Richmond Road, Bradford, West Yorkshire BD7 1DP, United Kingdom and Honorary Research Consultant to Bradford NHS Trust. Email:d.anderson1@bradford.ac.uk.

Dr Mojgan Najafzadeh MD, PhD post-doctoral fellow. Division of Medical Sciences, University of Bradford, Richmond Road, Bradford, BD7 1DP and Honorary Research Consultant

## 8.3 Appendix III

UNIVERSITY OF BRADFORD
MAKING KNOWLEDGE WORK

### 8.3.1 DATA COLLECTION FORM

(To be completed by the Doctor)

**STUDY TITLE:**

Genotoxic effects of nano and bulk forms of aspirin and ibuprofen on blood samples from prostate cancer patients compared to those from healthy individuals.

**REVIEWED BY LEEDS East RESEARCH ETHICS COMMITTEE (REC)**
**(REC REFERENCE NUMBER: (12/YH/0464)**

| | | | |
|---|---|---|---|
| PATIENT NUMBER | | DATE OF SAMPLE | |
| AGE | | | |
| SEX (PLEASE TICK) | M / F | CONSENT | Y / N |
| ETHNIC GROUP | | INFORMATION SHEET | Y / N |
| OCCUPATION | | | |

CURRENT SMOKER Y / N PAST SMOKER Y/N
CIGARETTES HOW MANY/MUCH PER Y /NDAY?
ALCOHOL Y / N CIGARS

| **DIET** | Western | Asian | OMNIVORE | VEGETARIAN | VEGAN |
|---|---|---|---|---|---|

| | |
|---|---|
| VITAMINS / ANTI-OXIDANTS (PLEASE LIST) | |
| PRESCRIBED DRUG USE (PLEASE LIST) | |

RECREATIONAL DRUG USE Y/N

IF YES PLEASE LIST

***MEDICAL***

Cancer inflammatory disease
Extent site

| | |
|---|---|
| CANCER | |
| Inflammation diseases | |
| Precancerous state | |
| Other medical conditions please list | |
| Family history of cancer and inflammatory disease | |
| Chemotherapy or radiotherapy | |

## 8.4 Appendix V

### 8.4.1 Solution for Comet and micronucleus assays

| Buffers/Reagents | Chemical constituents of reagents |
|---|---|
| Low melting point agarose (LMP) (storage at RT) | 0.5% (w/v) LMP in PBS |
| Normal melting point agarose | 1% (w/v) NMP in $ddH_2O_2$ |
| Final lysis solution | 89% lysis solution<br>100% DMSO<br>1% triton X-100 |
| Fresh electrophoresis buffer (storage at 4 $^oC$) | 1930 ml $ddH_2O$<br>60 ml 10 MNaOH<br>10 ml 200 mM Na2EDTA.$_2H_2O$<br>(pH 13.5) |
| Neutralising buffer storage at 4 $^oC$) | 12.11 Trizma Base pH 7.5<br>250ml $ddH_2O_2$ |
| Ethidium Bromide (EtBr) | 10mg/ml $ddH_2O_2$ |
| Final Ethidium Bromide solution | 20 µg/ml in $ddH_2O_2$ |
| Cytochalasin B storage at -20 $^oC$ | 10 mg of Cyto-B re-suspend in 2.5 ml of DMSO, diluted 1:4 in RPMI 1640 medium. |
| Phytohaemagglutinin storage at – 20 $^oC$ | 2mg PHA , 5ml RPMI 1640 medium |
| Basic Medium storage at - 4 $^oC$ | 84% RPMI 1640 medium<br>15%FBS, 1% Pen-Strep solution |

## 8.5 Appendix VI

### 8.5.1 Western Blotting: Solution preparations, Gel electrophoresis &Transblotting

**12% resolving gel**

| | |
|---|---|
| $H_2O_2$ | 6.6 ml |
| 30% acrylamide mix | 8.0 ml |
| 1.5 M Tris-HCl (pH 8.8) | 5 ml |
| 5.0 ml 10% SDS | 0.2 ml |
| ammonium persulfate | 0.2 ml |
| TEMED | 0.01ml |

**5% stacking gel**

| | |
|---|---|
| $H_2O_2$ | 6.8 ml |
| 30% acrylamide mix | 1.7 ml |
| 1.5 M Tris-HCl (pH 8.8) | 1.25 ml |
| 5.0 ml 10% SDS | 0.1 ml |
| ammonium persulfate 0.2 ml | 0.1ml |
| TEMED | 0.01ml |

**Preparation of solutions for western blot:**

**Tank Buffer: Add the following:**

Tris Base 30 g

Glycine 144 g

SDS 10g

Now make up to 1 litre of $dH_2O$

**Transblot Buffer 10X: Add the following:**

Tris Base 30 g

Glycine 144 g

Now make up to 1 litre of $dH_2O$

**Transblot Buffer 1X: Add the following for each run of western blot:**

10X Transblot buffer 100 ml

Methanol 100 ml

SDS 0.15 g

Fill up to 800 ml of $dH_2O$.

## 8.6 Appendix VII: Abstracts titles presented for Conference Contributions.

Azeza Guma, Adolf Baumgartner, Mojgan Najafzadeh, Mohammad Isreb, and Diana Anderson. **Genotoxic evaluation of nano- and bulk forms of aspirin and ibuprofen in lymphocytes from prostate cancer patients and healthy individuals.** UKEMS Annual Meeting, London, June 2016 (Poster).

2. Azeza Guma, Adolf Baumgartner, Mojgan Najafzadeh, Mohammad Isreb, and Diana Anderson. **Protective Effect of both nano and bulk forms of aspirin against tert-butyl hydroperoxide and bleomycin-induced oxidative stress in lymphocyte from prostate cancer patients and healthy individuals.** Molecular Epidemiology Group UK (MEGUK) Spring Meeting, Aberdeen, March 2017 (Poster).

3. Azeza Guma, Adolf Baumgartner, Mojgan Najafzadeh, Mohammad Isreb, and Diana Anderson. **Expression of p53, ATM and ATR after in-vitro treatment of lymphocytes from healthy individuals and prostate cancer patients with nano-sized and bulk forms of anti-inflammatory drugs**. UKEMS Annual Meeting, Leuven, June 2017 (Poster).